

# The dangers of coarse politics

**Keeping track of members of the US Congress is a good idea, but must be done with care. A science voting tally introduced by a group of eminent scientists does not fit the bill.**

THE publication last week of a Science Scoreboard, which rates each member of the US Congress on his or her voting performance on science and science-related issues, will probably have little discernible impact on November's congressional elections. The scoreboard may succeed, however, in exasperating a number of congressmen and women whose cooperation scientists can ill afford to lose.

The scoreboard has been produced by Science Watch, a group of eminent scientists and former senior science administrators, founded expressly to collate information of this type and pitch it into the public domain (see page 288). Science Watch believes that the old Washington ways of securing support for science — based on firm but discreet lobbying by scientific societies and universities in the Congress — are beginning to fail, and that the time has come to mobilize support for science directly at the grass-roots level. What better way to get the ball rolling than publicly rating the voting record of every member of Congress on science issues?

Science Watch selected 30 key votes in the House of Representatives as its basis for measuring members' commitment to science. Half of those votes concerned science budgets, and the rest dealt with a motley assortment of issues including the prohibition of certain types of research, barring individuals with financial interests at stake from peer review, and closing down parts of the science bureaucracy.

The results of that exercise is a ratings system that puts almost all Democrats at the top and almost all Republicans at the bottom. Texan Democrats Ken Bentsen, Sheila Jackson-Lee and Eddie Bernice Johnson each score 97 per cent under this system. But are their respective contributions really so much greater than those of champions of science on the other side, such as John Edward Porter (Republican, Illinois), saviour of the National Institutes of Health, who scores 38 per cent?

Science Watch says that it did not intend that the scoreboard would divide members so deeply along party lines, and was surprised at the outcome. But the design of its survey was always going to ensure such a result. The budget roll call votes, for example, by their very nature, are challenges to the Republican budget, likely to draw support chiefly from Democrats. The other votes could almost have been hand-picked to make Republicans fare badly.

Robert Walker (Republican, Pennsylvania), chair of the House Science Committee, and now the proud owner of a 40 per cent science scorecard rating, has responded vehemently to the exercise. Walker says he is frustrated that, after two years of stressing the importance of setting priorities, the science community still prefers "to take on the role of an entitled group". Walker's frustration is justified on this occasion. The community needs to demonstrate its understanding that it is Congress's job to make choices, and sometimes to cut programmes. The fact that the Republicans are now in political retreat does not make this any less true.

Whichever party controls the Congress after November's elections, science will face an uphill battle to maintain its share of the funding pie. The community has habitually underestimated its own strength, and the time is right for it to mobilize in defence of science. But before resorting to the megaphone, it needs to consider carefully what it wants to say: a refrain of "more money now" —

which is what this exercise amounts to — will not really suffice.

Science Watch says it has left the Senate alone, for now, because it has too few recorded votes on which to base a scoreboard. Before offending half the members of that august body, the group should apply some peer review to the validity of its own work, and perhaps ask itself whether the scoreboard, as applied by them, is too blunt an instrument for the operation in hand. □

## Charity begins abroad

**There are few virtues in being a poor relation, as researchers on diseases of the developing world know to their cost.**

POOR relations cannot expect substantial hand-outs when their richer relatives become increasingly preoccupied with domestic needs. Such is the case with the funding of research. At a time when government policies for research across the industrialized world are being explicitly moulded to two strategic objectives — increasing the wealth and quality of life of those who pay for it — the interests of others inevitably fall down the priority list. The French government is the latest to find cuts in research funding on issues relating to developing countries easier to absorb than cuts in its strategic priorities, for example nuclear and space technology.

A report published by the Wellcome Trust this week on the continued underfunding of research into what remains one of the world's most significant diseases, malaria, is therefore doubly welcome (see page 286). It provides some hard quantitative data about the relative expenditure on malaria compared to the more scientifically 'popular' diseases of the West, ranging from asthma to HIV/AIDS, demonstrating vividly the orders of magnitude that separate research efforts. And it is also a timely reminder that current trends in the science policies of Western governments are in danger of exacerbating this situation rather than reversing it.

The needs are certainly pressing. Another report, published jointly this week by the World Health Organization and UNICEF, emphasizes that the limited success of preventive measures and the spiralling rate of resistance to anti-malarial drugs both underline the urgency of an effective vaccine. But it also points out that clinical trials of candidate vaccines will be lengthy and costly until more research has been carried out on, for example, our understanding of immune responses. The disappointing results of trials of the Colombian vaccine Spf66, reported earlier this month, indicate that there seem to be few short cuts.

In an ideal world, malaria — as, indeed, other prevalent diseases in developing countries, such as leprosy and leishmaniasis — would receive as much scientific (and political) attention as HIV has done; certainly understanding the life-cycle of the parasite is as challenging as unravelling the behaviour of the AIDS virus. The former is far from neglected, witness the recent decision by the Wellcome Trust to sponsor the mapping of the *Plasmodium falciparum* genome, and other efforts to coordinate vaccine development. But, as most of those working in the field admit, much more could be done, even in the current state of knowledge. Poor relations never have an easy life. □



# Support for scientific evaluation of homeopathy stirs controversy

**Munich.** Horst Seehofer, the German health minister, last week gave a positive signal to supporters of homeopathic medicine, telling an international conference in Frankfurt that its success "cannot fundamentally be denied, even though this has often been attempted".

But he also told the meeting, held to celebrate the 200th anniversary of the publication of Samuel Hahnemann's description of the theory of homeopathy, that, like conventional medicines, homeopathic products "should always be able to prove their efficacy". His remarks coincide with the completion of a study carried

out for the European Commission (EC) which concludes that the efficacy of homeopathic products can indeed be proved using conventional methodology.

Under a directive on homeopathic remedies approved by the European Union in 1992, efficacy does not have to be demonstrated before homeopathic products are marketed. Moreover, homeopaths claim that their therapy cannot be adequately tested through conventional placebo-controlled double-blind clinical trials, as its success depends heavily on the sympathetic attention patients receive from practitioners.

But a year-long pilot study organized and funded by the EC at the request of the European Parliament, intended to indicate whether the EC should fund research into homeopathy, challenges this claim.

The ECU1-million (US\$1.25-million) study, which was formally completed last week, was carried out by a 16-strong group of experts, half sceptics and half proponents of homeopathy. At first, there was an apparently unbridgeable philosophical divide between the two groups. "The two blocks were very confrontational," says Jean-Pierre Boissel, a clinical pharmacologist at the University of Lyons.

But, according to Boissel, the group was surprised to find itself eventually reaching consensus on one of its most important questions, namely that there is no valid reason to exempt homeopathy from normal scientific rules of clinical and preclinical evaluation, provided the individual nature of homeopathic therapy is taken account of.

"We agreed that the efficacy of homeopathy can be tested according to the conventional guidelines for good clinical research," says Boissel. But, he says, "this not been

done yet", pointing to the group's analysis of more than 150 published clinical trials, most of which were judged to be of such low quality that no conclusions can be drawn.

The commission now faces the political decision whether to start European-level clinical trials as a result of the study. The issue is certain to be controversial, given the high level of opposition to homeopathy in pharmacological and medical circles. Flaminio Cattabeni, for example, professor of pharmacology at the University of Milan, Italy, and secretary of the European Federation of Pharmacological

Societies, says that such trials would be "a waste of money".

But the majority of both supporters and opponents of homeopathy favour more

scientific investigation of the subject, according to a survey conducted by the group of experts, which polled 1,500 doctors, medical researchers, health administrators, representatives of pharmaceutical and health insurance companies and politicians.

Homeopathy has an uneven pattern of acceptance in Europe. While it has never been widely accepted in Scandinavia and Britain, there is a high demand for homeopathic products in France, Italy and Germany. In Germany, insurance companies are even willing to pay for homeopathic treatment, despite steadfast opposition from the German scientific community.

But, as Ulrich Schwabe, professor of pharmacology at the University of Heidelberg, points out, insurance companies may be swayed by the fact that homeopathic products are relatively cheap. A final report of the findings of the EC group will be to the parliament before the end of the year.

**Alison Abbott & Gabor Stiegler**

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**Hahnemann: described theory  
of homeopathy 200 years ago.**

Wellcome Institute Library, London

## California doubles research tax credit

**San Francisco.** California's state legislature is trying to boost industry's spending on university research by approving significant increases in tax incentives for both basic and clinical research. The bill including the increases was approved by the legislature in early September, and is expected to be signed by Peter Wilson, the state governor, in the near future.

The bill applies to companies investing in research at a Californian university, research institute or university hospital. The state tax credit would double, from 12 to 24 per cent of eligible expenditures for basic research, and increase from 8 to 12 per cent of applied research spending. The credit will apply to any spending increases above a company's previous three-year average.

Enthusiastic support for the increased tax incentives has come from California's biotechnology industry. William Bold, director of public policy for the California Healthcare Institute, a lobby group for the biotechnology and pharmaceutical industry, says the credits are likely to inspire companies to put more money into basic research at universities and university hospitals in the state.

For the purposes of the law, 'basic' research is broadly defined to include both exploratory research and clinical tri-

als at university hospitals, which had not previously been eligible for tax credits. The credit may help companies to pursue basic research beyond a core of three or four lead products, Bold says.

Legislators hope that the increased credit — which will be higher than the tax incentives in any other state — will encourage biomedical companies to remain in California.

In the past, the University of California has not been welcoming to private investors, according to David Nagler, director of state affairs for Genentech. He said the company hopes the university will use the credit to attract more contracts for basic and clinical research. The state franchise tax board has estimated that Californian biotechnology companies spend almost \$20 million on university clinical trials within the state. With the tax credit, that figure should double, the board said. "I hope that's wildly conservative," Nagler added.

The use of tax incentives to boost industry research spending remains controversial. Nevertheless, a report prepared by the accountants KPMG Peat Marwick LLP concluded that a \$1 reduction in after-tax cost of research creates about \$1 of additional spending initially, and \$2 in the long term. **Sally Lehrman**

# Science rises up US space policy agenda

**Washington.** The White House last week released a new US space policy — the first produced since the end of the Cold War — which includes several specific goals for science, including a systematic study of Mars by robots and a long-term programme to search for planets around other stars.

The document, which mostly codifies Clinton administration policies and activities already under way, is the first complete overhaul of the nation's space policy since 1989. The commitment of the previous Bush administration to a human landing on Mars has been replaced by a wait-and-see approach. According to a fact sheet distributed last week, the space station will now merely "support future decisions" on human exploration of the solar system.

The new policy calls for human and robotic exploration of space, as well as "a sustained programme to support a robotic presence on the surface of Mars by year 2000 for the purposes of scientific research, exploration and technology development".

The National Aeronautics and Space Administration (NASA) already has plans to

send spacecraft to Mars at every two-year launch opportunity, beginning this fall. But those plans are being reconsidered in light of a recent claim of past biological activity on Mars (see *Nature* **382**, 565; 1996). White House press secretary Michael McCurry told reporters last week that the new policy envisions a "continual transmission of data from the surface of Mars" by 2000.

Science has a higher billing than it did in the 1989 document, which listed national security as the top goal of the US space programme. Now the first goal is to "enhance knowledge of the Earth, the solar system and the universe". The policy is also more specific than its predecessor about which scientific programmes NASA will undertake.

Aside from exploring Mars, it calls for the agency to conduct long term programmes to "obtain in-situ measurements and sample returns from the celestial bodies in the solar system", and to "identify and characterize planetary bodies in orbit around other stars". The latter goal is a major part of the space agency's proposed new 'Origins' programme (see *Nature*, **378**, 650; 1995).

The policy strengthens the administration's commitment to Earth observation from space, including continual measurements from the Earth Observing System — which came under attack last year from the Republican-led Congress — by 1998. It emphasizes low-cost spacecraft, directs NASA to buy spacecraft and data wherever possible from the private sector, calls for the eventual transition from a regulated market for international launches to one that is free and open, and encourages greater cooperation and cost-sharing between civilian and defence space sectors.

According to Ray Williamson, an analyst at the Space Policy Institute at George Washington University, the new policy also "makes a stronger statement on international cooperation". Past US language on international projects such as the space station always had a slightly "America-first" tone, he says. Now the United States promises to pursue "greater levels of partnership and cooperation in national and international space activities".

**Tony Reichhardt**

## Malaria research suffering relative neglect, study claims

**London.** Malaria research is drastically underfunded compared to other diseases, such as HIV and asthma, when its relative incidence and its global death toll are taken into account. That is the conclusion of a report published this week by the Unit for Policy Research in Science and Medicine (PRISM) of Britain's Wellcome Trust. The report\* also suggests the results of research have not been sufficiently exploited.

Malaria kills between 1.5 million and 2.7 million people a year, particularly in sub-Saharan Africa; other regions with major problems include India, South America and the Far East. The prevalence and distribution of the disease is increasing. The *Anopheles* mosquito vector is gaining greater resistance to insecticides, and the principal infectious agents, *Plasmodium falciparum* and *P. vivax*, are becoming more resistant to antimalarial drugs.

The PRISM analysts looked at global expenditure on malaria research over the past decade, and associated international publishing activity. They asked 200 members of the malaria community — including researchers, clinicians, health service personnel, industrialists and administrators — about their views on current practice, and the most useful directions for research.

"We've identified the scale of the difference of investment between malaria and other diseases," says Joe Anderson, one of the report's authors and the director of the unit. Total global expenditure on malaria research in 1993 was US\$84 million —

equivalent to \$42 for every death. Calculations for HIV/AIDS gave \$3,274 for each death, and \$789 for asthma.

According to Anderson, these figures confirm how research funding places a disproportionate priority on diseases with a high profile in the West. It has been received wisdom that tropical diseases get less attention than others, he says, but PRISM has now produced "proper facts and figures that people can use to make policy decisions".

The situation appears to be getting worse. Global funding for malaria research declined significantly between 1985 and 1994, largely reflecting a drop in US investment as it reduces its military involvement overseas.

Nevertheless, more than half of all funding in 1993 still came from the United States, particularly the US Agency for International Development and the National Institute of Allergy and Infectious Diseases.

The report shows that international publishing activity reflects the relatively low funding. While Britain's global share of publications increased from 14 per cent in 1984 to 18 per cent in 1994, that of the United States fell from 42 to 34 per cent.

Some 115 members of the international malaria community responded to the opinion survey. Two-thirds of them had had personal experience of projects in which findings led to improvements in disease prevention, treatment or control, suggesting that research appears to have a

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**Malaria victim: research funding is losing out.**

direct impact on health care.

Yet half said they were aware of promising research findings that have not been fully developed in fields such as potential drugs and vaccines, the treatment of severe and cerebral malaria, biological control of the disease vector and the monitoring of drug resistance. "That's an exploitation gap," says Anderson.

Obstacles to better exploitation, according to the survey, include poor orientation of research programmes to practical problems and public needs. Topics identified as having the best prospects for advancing understanding over the next five years were the genetics and biology of *Plasmodium* and disease epidemiology.

**Claire O'Brien**

\*Anderson, J., Maclean, M. & Davies, C., PRISM Report No 7. *Malaria Research: An Audit of International Activity*. (Wellcome Trust, London, 1996, £10.)



## Republicans challenge evidence of man-made climate change

Washington. A group of Republicans in the US Senate has told the Clinton administration that they remain suspicious of the scientific evidence for man-made climate change. They oppose any international agreement that would require the United States to limit emissions of greenhouse gases.

Views expressed at a hearing of the Senate Energy and Natural Resources Committee last week indicated that, if Clinton is re-elected in November and he attempts to negotiate a binding international agreement on greenhouse gas emissions, many minds will need changing before the deal can win Senate ratification.

Frank Murkowski (Republican, Alaska), the committee's chairman, said that "the scientific jury is still out over the question" of whether human activities are likely to result in "dangerous, significant, adverse climate change".

Murkowski asked whether the administration, by backing the concept of a binding international agreement on greenhouse gas emissions, was "trying to achieve through the United Nations something it had failed to achieve before Congress".

He repeated the allegation that the recent assessment of climate change by the Intergovernmental Panel on Climate Change (IPCC) had been "heavily edited after what was apparently pressure from policymakers". This claim was first made by the Global Climate Coalition, an industry-backed lobby group.

But Bennett Johnston (Louisiana), the senior Democrat on the committee, jumped to the IPCC's defence. He called the charge "a disgrace", adding that "there is not a shred of evidence of misconduct by any of the scientists involved". Johnston, who has strong ties to the oil and nuclear industries, said that "industry should stop damaging its own credibility by attacking the IPCC report".

Timothy Wirth, under-secretary for global affairs at the State Department, testified that the charges against the IPCC "had been totally refuted by the scientists involved". He added that the Department of Commerce was leading an inter-agency team studying the economic effects of different climate change policies.

Wirth said that industry was being asked to participate in this process, which would help to establish the US position in negotiating an international agreement. But the retirement in November of Johnston — a conservative Democrat widely respected on both sides of the Senate — will make more difficult the task of ratifying such an agreement, even if it is reached. C. M.

## Cash rivalry 'fosters neglect of postdoc supervision'

Washington. The supervision of postdoctoral students is being neglected by supervisors who fear that the students will become rivals for a diminishing 'pot' of research funds, an international meeting at George Washington University (GWU) in Washington, DC, was told last week.

"We are seeing a considerable drop-off in mentoring because [the supervisors] perceive the youngsters as competitors," Julian Jack, deputy chairman of the Wellcome Trust, told the meeting, held to discuss a perceived 'crisis in science'.

Jack promised that the London-based trust — one of the world's largest private sources of medical research funding, distributing over £200 million (US\$300 million) last year — would act as a "kind of postdoc liberation front", ensuring fairer treatment for the postdoctoral students funded by its grants.

His point was echoed by Robert Pollack, a professor of biology at Columbia University, New York, who recently closed his laboratory to concentrate on teaching. Pollack said that many scientists were asking why they should bother to help a postdoc to get started as an independent scientist if they were shortly going to be competing with that person for funds. He said that, as a result of a "diminished and cynical view of human relations, the education of many young scientists is deeply defective".

The meeting, 'Science in Crisis at the Millennium', which was organized by Horace Freeland Judson, director of GWU's new Center for History of Recent Science, heard of an unfolding crisis across the scientific community. Speakers said the problem was rooted in lack of money to continue the rapid expansion to which the system had become accustomed.

Diminishing civility and 'collegiality' among researchers, the growing number of postdocs competing with their professors for funds, the increased politicization of peer-review panels and lack of effective self-regulation of misconduct were all put forward as what Judson calls "signs and symptoms" of a "deeper malady" in science.

But not everyone agreed these symptoms amount to a crisis. Jack Gibbons, science adviser to President Bill Clinton, admitted that the scientific community faces "enormous challenges" in adjusting to slower funding growth and changing public expectations. But a true crisis, he said, would be approaching the limits of knowledge. "We shouldn't worry; we are quite secure in our ignorance."

The gloomiest prognosis came from Pollack, who argued that a "crisis of scientific morale" has gripped molecular

biology, despite continuing productivity and a relatively healthy funding outlook.

"Low morale is not a matter of too little money, nor of too few ideas, but of too little kindness and decency," said Pollack. "It is a failure of custom and manners, a loss of social purpose, a diminution in the ability or the will to distinguish right from wrong and then to act rightly."

Pollack said he shut his laboratory because he was tired of "spending time pawing through the results of expensive, careful experiments just to find bits of bait to put on the hook of another grant application". For most scientists today, he said, "the job is to get the grant"; everything else was a "distraction".

John Ziman, emeritus professor of physics at the University of Bristol in the United Kingdom, argued that sharp changes



in the language used to discuss science in the past 25 years indicated "a general state of crisis, pervading the whole enterprise from top to bottom". Reeling off a list of the offending words — accountability, allocation, appraisal, career, centre, competition, contract, critical mass — Ziman said that "each of them comes trailing clouds of red tape and disappointment". He pointed out that few were used in this context in 1970. "It's a very big change in such a short time."

The change had come about because the resources available for science, which had been doubling approximately every 15 years for 300 years, "could not conceivably double again". Other problems, Ziman said, "are supplemental to that single fact".

Ziman argues that a new paradigm of "post-academic science" is emerging as a consequence, in which scientists will not normally have tenure, and will engage in projects at the behest of government agencies and industry (see *Nature* 382, 751; 1996). These scientists would have "neither examples of disinterested behaviour to emulate, nor examples of objectivity to live up to", he warned.

Colin Macilwain

## NIH edges towards healthy increase in research budget

Washington. Congressional negotiators reportedly agreed late last week to grant the National Institutes of Health (NIH) a 6.9 per cent increase in funding in the 1997 fiscal year, which begins on 1 October.

The \$819-million increase, which would result in a total NIH budget of \$12.7 billion next year, was being negotiated as part of a broad spending bill covering those parts of the US government for which individual spending laws have not been enacted.

Congress was expected to vote on the omnibus spending bill as soon as Tuesday or Wednesday of this week. Legislators are keen to adjourn so that they can return home to campaign for the congressional elections that are due to take place in November.

If the generous NIH increase is approved, it will represent a victory for the backers of biomedical research, given current government austerity.

"We are cautiously optimistic," said John Suttie, a biochemist at the University of Wisconsin-Madison, who is president of the Federation of American Societies for Experimental Biology, responding to reports that negotiators from the House of Representatives and the Senate had agreed on the 6.9 per cent increase.

The new funding shows that biomedicine backers and their chief House advocate, John Porter (Republican, Illinois), have been "tremendously successful", said Peter Kyros, a lobbyist for the Joint Steering Committee, a group of five societies representing laboratory scientists.

The 6.9 per cent figure was first approved by the House of Representatives in a bill funding the Departments of Labor, of Health and Human Services and of Education. That bill passed the House in July. But the Senate Appropriations Committee this month backed a lower, 4.1 per cent NIH increase in its own, parallel bill.

That increase, of \$487 million over 1996, would have resulted in a 1997 NIH budget of \$12.4 billion. This figure compares with the 3.9 per cent increase over 1996 funding requested by the Clinton administration in March.

Senate negotiators are said to have been able to meet the House figure because congressional Republicans had accepted a White House demand that Republicans restore between \$5.1 billion and \$6.5 billion in domestic spending that Clinton requested in his March budget proposal.

Meredith Wadman

## Democrats score highest in 'votes for science' review

Washington. Three Texan Democrats are the most stalwart supporters of science in the US House of Representatives, according to a scorecard compiled by Science Watch, a new lobby group, based on 30 key science-related votes over the past two years.

Ken Bentsen, Sheila Jackson Lee and Eddie Bernice Johnson each voted "for science" 97 per cent of the time — 29 of the 30 votes — earning them top place in Science Watch's league table. Overall, Democrats voted in favour of science twice as often as Republicans.

Science Watch was set up by a bipartisan group of science leaders, including Roland Schmitt and James Duderstadt, both former chairmen of the National Science Board, several Nobel laureates, and Allan Bromley, science adviser to George Bush, the last Republican president.

Having selected the 30 key votes from a shortlist of 100 science-related votes in the 104th Congress, the group found a massive gulf between Republican and Democrat members. Democrats voted for what Science Watch took to be the "pro-science" position 70 per cent of the time, against a Republican average of 35 per cent.

"We didn't expect the party difference," says Martin Apple, executive officer of the Council of Scientific Society Presidents, and chief executive of the watchdog organization. "The degree to which we found it was extraordinary."

Republicans have reacted angrily to the findings, rejecting Science Watch's choice of criteria. "The bottom line of this survey is that, if you're a big spender, you get an 'A'," says Robert Walker (Republican, Pennsylvania), chairman of the House Science Committee, who got a rating of 40 per cent. Walker fired off a letter to the lobby group,

saying: "I hope that in future you will review your methodology and not succumb to the temptation to further politicize science."

But Apple says that the scorecard did not necessarily favour big spenders, as the budget votes which it considered generally involved a transfer of money between science agencies and other accounts, rather than additional spending. The scorecard included 17 such money-related votes, but also votes on the prohibition of certain types of environmental and embryo research, on peer review, science education and the elimination of certain science agencies.

Walker says the list ignores Republican actions to secure funding for basic research. He describes the inclusion of the embryo question as "a cheap shot, since the decision of most members on that vote was a matter of conscience, not a question of science".

Other Texan Democrats also scored more than 90 per cent in the survey, as did John Murtha (Democrat, Pennsylvania) and George Brown (Democrat, California). Republicans who fared best in the exercise, scoring more than 50 per cent, included Tom Davis (Virginia), Sherwood Boehlert (New York), Connie Morella (Maryland) and Vernon Ehlers (Michigan).

Scorecards of this type are often kept by special interest groups in the United States, and used in a none-too-subtle manner to influence elections. But the publication of such a list by an eminent group of scientists before November's congressional elections will, reopen the question of whether the science community has either the muscle or inclination to operate in this realm.

Schmitt, who constructed the survey with Apple, conceded that its publication was risky. "Not everyone will agree with our approach," he says.

Colin Macilwain

## Call for regional research boost in Europe

**Brussels.** The spending guidelines for the European Union (EU)'s so-called 'structural funds', which are intended to reduce the gap in competitiveness between rich and poor regions of Europe, should be changed to allow more money to be used to strengthen the research base of member states, a senior official of the European Commission said last week.

Eneko Landaburu, director general of the commission's regional development and cohesion directorate, was speaking at the third Regional Science and Technology Policy Research (RESTPOR) conference hosted by the commission in Brussels. He said that 7 per cent of

structural funds allocated so far — equivalent to ECU7 billion (US\$5.6 billion) — had been spent directly on improving the infrastructure for research and development (R&D) in "less favoured" regions.

But this is not enough, he said, given the links that exist between research and competitiveness. Landaburu criticized the governments of the EU's member states for not giving priority to research in their applications for structural funds, part of which are also known as cohesion funds. Governments preferred to emphasize more visible projects, such as road-building, with greater appeal to voters.

Alison Abbott

# French researchers brace for budget cuts

**Paris.** For the first time in ten years, the French government is proposing to decrease the level of funding for civilian research. Proposals submitted to parliament last week envisage a reduction of 1.4 per cent next year — or 3.3 per cent in real terms, taking inflation into account — from FF53.1 billion (US\$10.3 billion) to FF52.3 billion (US\$10.2 billion).

This proposed reduction will have relatively little impact on publicly funded research establishments, and has so far generated relatively little reaction from researchers and their unions, partly because it was not unexpected. But proposals from the state secretary for research, François d'Aubert, to tackle the problem of recruiting young researchers have done little to allay concern among scientists still reeling from a freeze imposed last year.

Funding for European-level laboratories, such as the European Molecular Biology Laboratory and the European Southern Observatory, will be reduced by a total of 3.14 per cent. This echoes a similar decision by the German government in late July (see *Nature* **382**, 285; 1996). France's contribution to the European Laboratory for Particle Physics (CERN) is likely to be reduced by about 2 per cent to FF700 million, according to officials.

Faced with a need to reduce the public deficit, Alain Juppé, the prime minister, has decided to achieve this through cuts in government spending. Total spending will now be FF1,553 billion next year — the same in current francs as last year, despite an expected rate of inflation of 1.9 per cent. In recent years government spending has been increasing by 4 per cent a year on average.

Almost all government ministries have had to make cuts. But a few have been spared, and the Ministry of National and Higher Education, which has responsibility for research, is one. With a total budget of FF324.2 billion, it will benefit from an increase of just over 2 per cent.

Research will not share this overall growth. Nevertheless, Dominique Poiroux, d'Aubert's chief of staff, says that it remains a high priority for the government. He points out that the reduction in the research budget will primarily fall on industrially related research and aeronautics — which will receive FF360 million less than this year. Many of the research programmes in aeronautics have been completed.

In industrial research, new initiatives may offset the loss of funds. ANVAR, the agency responsible for innovative companies, will see its budget reduced by FF150 million, but the creation of 'investment funds' along American lines will help to divert private capital to such companies.

"The important thing is to preserve publicly funded research, and the operating

resources of our laboratories," says Poiroux. He adds that support for programmes, which, when combined with incentive funds and equipment funds, make up laboratory resources, should increase by 3.1 per cent compared to 1996 for government research establishments.

But as far as their overall budgets — including staff costs — are concerned, not all government laboratories are in the same boat. The budget of the Centre Nationale de la Recherche Scientifique (CNRS), the main supporter of basic research, will grow in current francs to FF13.45 billion, an increase of 1.12 per cent. That of the medical research agency, INSERM, will stay the same as last year, while ORSTOM, which is responsible for research in developing countries, will see its budget fall by 1.8 per cent.

In contrast, bodies responsible for national strategic priorities — space and nuclear — will have their budgets maintained, with a slight increase for the Atomic Energy Commission. The space agency CNES will as a result be able to meet

its international commitments, in particular to the space station, and to pay its share of the costs resulting from the loss of the first Ariane-5 launch.

University research, for which d'Aubert has often expressed concern over its relatively poor performance, also turns out to be one of the winners. Despite the overall budget austerity, it will receive 3.15 per cent more than last year.

D'Aubert wants to address the problem of the recruitment of researchers head on. "Research bodies are being strangled by their staff costs, which are continuing to grow at the expense of operating funds," says Poiroux.

The government's solution appears simple: the rate of recruitment will be fixed at 2.5 per cent of the total staff number, and measures will be introduced to encourage researchers to take up posts in universities or industry. If more positions are freed than there are recruits, the excess posts will be eliminated, and the money used for operating costs.

**Catherine Tastemain**

## Swiss cull to meet fears of BSE

**Basel.** Switzerland's government announced last week that it has decided to subsidize the slaughter of 230,000 cows born before 1 December 1990 to restore the faith of consumers in Swiss beef. Feeding calves with animal protein, considered to be responsible for transmitting bovine spongiform encephalopathy (BSE), was legal until 1990.

Farmers will receive SF1,000 (US\$800) compensation for each cow slaughtered, and their use for human consumption will be banned. "This move is intended to restore to Switzerland the status of a BSE-free country," said the economics minister, Jean-Pascal Delamuraz.

BSE has already caused a serious economic problem for Swiss farmers. The domestic market has fallen by a third since the beginning of the year, leading to reduced beef prices and the decision to store large quantities of meat. Foreign markets have also been blocked, as the European Union — of which Switzerland is not a member — has banned the import of Swiss beef. The ban followed an announcement in March by the British government of a possible link between BSE and Creutzfeldt-Jakob disease in younger patients.

Switzerland has the second highest incidence of BSE in Europe, although, at 9.5 cases per 100,000 cattle, this is only one-hundredth of the UK level. "We intend to remove from the human food chain the entire cattle population which has a statistical chance of harbouring BSE-infected animals," says Heinz Mueller, a spokesman for the federal veterinary agency.

IMAGE  
UNAVAILABLE  
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REASONS

**Better beef? The Swiss slaughter focuses on cows that may have been fed animal protein.**

According to Mueller, the recent study by Roy Anderson and his colleagues at the University of Oxford showing that culling is likely to have only a limited impact on the extinction of BSE in the United Kingdom (see *Nature* **382**, 779; 1996) is not relevant to Switzerland, as the objectives of the two countries are very different. "We want to rebuild trust in Swiss beef as soon as possible," he says.

The remains of Swiss cows born before 1990 will be used in animal feed given to pigs. But this is likely to cause problems. The two leading supermarket chains, Migros and Coop, announced last month that, in order to restore public trust in their products, they will stop selling meat from any species that is raised on feed containing animal protein.

**Oliver Klaffke**



# Galapagos tortoise disease 'contained'

**Paris.** Researchers and conservationists said last week that they have contained the outbreak of a mystery disease that has killed eight giant tortoises and affected nine others on the Galapagos island of Santa Cruz.

But the cause of the deaths has yet to be established. And the outbreak has reopened a stormy debate over the potential threat to the Galapagos ecosystem from human activity, including agriculture.

About 20,000 giant tortoises live in the Galapagos archipelago. The deaths, which were first reported last month, have all occurred in and around a large pond in El Chato, a region within the Galapagos National Park that is open to visitors.

The pond is one of the few waterholes in the area to have survived recent droughts, and has attracted many tortoises, as well as cattle, goats, pigs, dogs and other animals from a nearby farming region.

The tortoises' symptoms include general

fatigue, secretions from the eyes, nose and mouth, and difficulty in breathing, with the exhaled air having a putrid odour. A similar outbreak in the same area in 1979 killed five tortoises, but it was detected too late for samples to be taken from the already decomposed animals.

"When animals die in the field you have to be very lucky to get one fresh," says Elliott Jacobson, a professor of zoological medicine at the University of Florida, who heads the team studying the pathology of the new outbreak. "Many changes that occur in tissues shortly after death mask everything."

This time, blood and faecal samples were taken from several animals immediately after they died by staff at the Galapagos National Park Service and the Charles Darwin Research Station. The research

IMAGE  
UNAVAILABLE  
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REASONS

**Tortoises at risk: human activities may be a factor.**

station is part of the Charles Darwin Foundation, an international nonprofit organization set up in 1959 to carry out research in the Galapagos Islands.

Such an operation in this remote area would also have been impossible before the advent of electronic mail, says Jacobson. "Tissue collection and handling is very complicated," he says, and it is impractical "to have a team standing around" because outbreaks occur rarely.

E-mail allowed Jacobson and other scientists to coordinate with staff in the Galapagos to develop protocols with the limited materials and reagents they had in hand or were able to obtain from the Gustavo Orces Herpetological Foundation in Quito on the mainland of Ecuador.

Nevertheless, what killed the tortoises may be established, if at all, only after a long and tortuous investigation. Establishing the cause of death in wild mammals is difficult enough, but it is relatively straightforward compared with the problem in reptiles, where the field is in its infancy. Jacobson, an expert on tortoise diseases, points out that diseases in desert and gopher tortoises are probably the best understood among those of reptiles, but that even in these cases "we still don't understand the dynamics".

As for the Galapagos outbreak, pathological studies may point the investigation in the right direction, but for now it is not even known whether the disease is infectious.

The working hypothesis is that it may have resulted from a combination of factors. The tortoises have taken to eating introduced passion fruit, for example, which may have reduced the nutritional value of their diet and made them susceptible to usually innocuous threats, such as nematode infections, algal blooms or pesticides.

Although agriculture has not yet been identified as the culprit that caused the outbreak, suspicion that this is the case has reopened the debate over the risks posed to the Galapagos ecosystem by human activities, in particular because the pond where the outbreak occurred is shared by farm animals.

**Declan Butler**

## Appeal to reprieve Canadian cyclotron

**Ottawa.** More than 700 scientists from 37 countries have appealed to Canada's prime minister to save the Tandem Accelerator Superconducting Cyclotron Facility (TASCC) at Chalk River Laboratories, the country's only heavy-ion research centre.

The appeal has been made in the form of a paid advertisement in the Toronto newspaper *The Globe and Mail*. Signatories include six Nobel prizewinners, the presidents of six national physical societies and the directors of ten major world laboratories.

TASCC is under threat following a decision by the federal government last March to cut nearly C\$75 million (US\$55 million) from the C\$175 million budget of Atomic Energy of Canada Ltd (AECL) (see *Nature* **380**, 92; 1996). Both the cyclotron facility and the condensed-matter science programme were given until next March to find alternative funds.

D. Allan Bromley, the former US presidential science adviser, describes TASCC in the advertisement as "the best such anywhere in the world today and I think I have seen them all". Sir Denys Wilkinson, former vice-chancellor of the University of Sussex in the United Kingdom, is quoted as saying in a letter to Anne McLellan, the minister of natural resources, that there are "very few areas in respect of which Canada occupies an uncontested place at the top table; nuclear structure physics through TASCC is one of these".

Despite receiving more than 200 letters of support for TASCC, McLellan

has said for the past six months that she is too busy to meet representatives of the cyclotron facility, according to John Hardy, its director. Other senior government ministers say they cannot help without McLellan's approval.

"The [open] letter [tactic] was chosen because we don't think we have the government's attention," says Hardy. The National Research Council of Canada is known to be interested in taking over the project, but does not have funds available.

The Aerospace Industries Association of Canada has indicated its interest in discussing a possible future partnership. TASCC already does nearly C\$1-million worth of business annually with aerospace industries.

The most telling pressure for TASCC funding may come from the town of Deep River, near which AECL is located. This small community is the only one in Canada that has agreed to the nearby burial of low-level nuclear waste from Canada's reactors — something the federal government is anxious to see.

But the town's agreement contains a condition: that the number of AECL jobs will not fall from its level in March last year. It is already 200 jobs below that, and the disbanding of TASCC and the condensed-matter science programme would increase the job losses by a further 100. These would include scientists with expertise in nuclear-waste disposal. The government could lose the town's approval for nuclear-waste disposal if the jobs are lost.

**David Spurgeon**



# Japan's HIV blood scandal broadens out

**Tokyo.** The president and two former presidents of Green Cross Corporation (Midori Juji), Japan's leading blood product manufacturer, were arrested last week in connection with the infection of thousands of Japanese with HIV through the use of non-heat-treated blood products in the 1980s.

Further arrests are expected, possibly including a former official of the Ministry of Health and Welfare, as attention in Japan broadens out from a previous focus on haemophiliacs who had been infected with HIV to non-haemophiliacs.

Those arrested last week are the current president of Midori Juji, Takehiko Kawano, and two of his predecessors, Renzo Matsushita and Tadakazu Suyama. They are being held on suspicion of negligence in promoting the continued sale of non-heat-treated blood coagulants in 1986, after safer heat-treated products, including those of the Midori Juji company, were approved for sale by the government in 1985.

The three arrests relate to the death of a non-haemophiliac who was treated with the company's blood coagulants during 1986 for a liver ailment. This has drawn attention to the fact that, as well as haemophiliacs, thousands of non-haemophiliacs were treated with blood

products potentially infected with HIV.

Documents confiscated from the company by prosecutors in Osaka are said to include an internal company memorandum sent in January 1986 to employees by Renzo Matsushita, then president, claiming that the company's non-heat-treated products were safer than heat-treated ones, as they were made from domestic blood. But in fact nearly all of Midori Juji's blood products were made from blood plasma imported from the United States.

Prosecutors suspect that the three executives continued to promote the sale of non-heat-treated products in 1986, despite knowing the risk of HIV infection and the availability of safer heat-treated products.

According to newspaper and television reports, Midori Juji executives were repeatedly warned of the dangers of HIV contamination of non-heat-treated products by the company's US subsidiary as early as 1984. But they failed to take any action.

The company was very slow to withdraw non-heat-treated products, leaving them on the market until 1988 — two years after the company had reported to the Ministry of Health and Welfare that withdrawal had been completed (see *Nature* 380, 472; 1996). As a result, many Japanese may have been infected with HIV several years

after the dangers of non-heat-treated products were realized.

Only this year did it become widely known that thousands of non-haemophiliacs had been treated with the non-heat-treated products (see *Nature* 379, 483; 1996). Despite a belated attempt by the ministry to trace patients treated with the products, most have not been located because hospitals usually destroy patient records after five years, and patients in Japan are seldom told what they are treated with.

Nevertheless, several HIV-infected individuals have been tracked down. Now that haemophiliacs have received large compensation settlements from the government and blood-product manufacturers, attention is switching to non-haemophiliac victims of the affair.

Ministry officials face possible criminal charges for failing to order the withdrawal of non-heat-treated products. These may include Akihito Matsumura, who headed the ministry's biologics and antibiotics division from 1984 to 1986.

Last week, the family of the same victim as in the Midori Juji case filed a charge of murder against Matsumura at the Osaka District Prosecutor's office, as they had done earlier this year against Matsushita.

David Swinbanks

# UK chief scientist warns of risks of 'Ig Nobel' ridicule

Boston. The organizers of the annual unofficial 'Ig Nobel' prizes — due to be awarded for the sixth time at Harvard University in Cambridge, Massachusetts, next week — have been warned by Robert May, the British government's chief scientific adviser, that ridiculing genuine scientific projects can be counter-productive.

May expressed his concerns in a recent interview with *Nature* and in letters sent to Marc Abrahams, editor of the journal *Annals of Improbable Research* and chief orchestrator of the annual proceedings, and Harvard physicist Sheldon Glashow. He explains his concern on the grounds that last year's award of the Ig Nobel physics prize to scientists at the UK Institute of Food Research in Norwich for "the study of the effects of water content on the compaction behaviour of breakfast cereal flakes" had "caused a lot of grief".

After receiving the award, the scientists were pilloried in the British press and on television. May, who is Royal Society research professor at the University of Oxford, says he is all in favour of light-hearted fun, and acknowledges that research on the "physics of snap, crackle, and pop" does sound humorous. "But it's

actually serious work by a serious research group," he says.

In future, May suggests, the directors of the ceremony should secure the permission of prospective recipients, to avoid damaging science in the way that Senator William Proxmire's Golden Fleece awards once did.

The point of the Ig Nobel prizes, he says, should be to strip science of some of its self-importance, and not to hold scientists up to ridicule. "I most earnestly hope that you will revert to attacking those areas that deserve attack, particularly in anti-science and attack pseudo-science, while leaving serious scientists to get on with their work," he asserts.

Abrahams says that he basically agrees with May. "We go to a lot of trouble to contact scientists and get their consent," he says. "The Norwich scientists were willing participants, even to the extent of

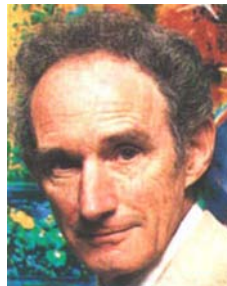
sending us a funny videotaped acceptance speech."

What happened afterwards, he says, was not anticipated and regrettable. But on balance, he says, the Ig Nobel prize ceremony does more good than harm. "Most of the great breakthroughs in science were once regarded as funny and unimportant. We believe that it's good to pursue ideas that sound funny and see where they lead."

Glashow — the Nobel prizewinning physicist who has participated in several Ig Nobel events — supports Abrahams' view that the ceremony has had a positive effect. "It increases awareness that science can be fun," Glashow says. "Contrary to the stereotype in movies, scientists can enjoy their work and do, in fact, have a sense of humour. At least most of them."

Ten prizes will be awarded at this year's ceremony in Harvard's Sanders Theater. Plaster casts of the feet of actual Nobel laureates will be auctioned, the traditional Heisenberg Uncertainty Lectures will be aired, and the world premiere will take place of *Lament del Cockroach*, a mini-opera for Nobel laureates and mezzo sopranos.

Steve Nadis



May: awards should in anti-science and attack pseudo-science.

## Fair wind for renewable energy in Germany and beyond

**Munich.** Angela Merkel, Germany's environment minister, last week told an international solar energy conference in Freiburg that the contribution of renewable resources to Germany's energy balance should double from 2.5 to 5.0 per cent of the total by the year 2005. This would enable solar energy, as well as wind, water and biomass energy, to contribute to the 25 per cent fall in total carbon dioxide emissions to which Germany is committed by that date, she said.

Meanwhile, a report from the Worldwatch Institute in Washington says that wind power is growing more rapidly than any other source of energy. It estimates that, at the end of 1995, the world's 25,000 wind turbines were generating 4,900 MW of electricity per year. The global capacity represents a 30 per cent increase on 1994. □

## Planning for the APE1000

**Munich.** Germany's national research centre for particle physics, DESY, and Italy's National Institute for Nuclear Physics, last week signed an agreement to build a next-generation massively parallel computer by the year 2000. To be called the APE1000 (Array Processor Experiment, 1000 gigaflop per second), the computer will be ten times faster than the current APE100. It will be installed at DESY's Institute for High Energy Physics in Brandenburg. □

## Los Alamos director to quit

**San Diego.** Siegfried S. Hecker announced last week that he is to resign as director of the Los Alamos National Laboratory in New Mexico, a post he has held for almost 12 years, with effect from October 1997. According to officials at the laboratory, Hecker is

leaving to pursue new professional challenges, but will remain a senior fellow at the laboratory. He has also been asked to advise officials at the University of California, which runs the laboratory. According to Richard C. Atkinson, president of the University of California, the laboratory has achieved under Hecker's stewardship "great acclaim as one of the world's premier research institutions". □

## Production for 'herbal' petrol

**New Delhi.** Ramar Pillai, who claims to have invented a 'herbal' petrol (see *Nature* 383, 112; 1996), is going into business. The state government of Tamil Nadu has given him 20 acres of land to grow the herb, and permission to produce the fuel in his basement factory, which has been given security protection.

Pillai will produce 50 litres a day, increasing to 300 litres a day after six months. The so-called Western Ghats Oil, named after the hills where the herb was found, will be sold at 10 rupees (30 US cents) a litre, about half the price of conventional petrol. Meanwhile, India's Department of Science and Technology is hoping to have patented the invention by the end of next month. Tests at the Indian Institute of Petroleum in Dehra Dun are reported to have confirmed earlier findings that the herbal fuel can act as a petrol substitute. □

## Growth for UK universities

**London.** The number of British entrants to full-time undergraduate courses in UK universities is likely to grow by about 5 per cent a year until 2000, with continued growth in part-time, international, post-graduate and non-degree students, according to a report published this week by the Institute for Employment Studies.

The report, *University Challenge: Student Choices in the 21st Century*, was commissioned by the Committee of Vice-Chancellors and Principals. It points out that the 1.6 million students in 115 universities and 68 higher education colleges represents a doubling

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of student numbers over the past 25 years. Of 200,000 students entering part-time study in 1994, one-third were at postgraduate level, which has experienced a rapid growth in part-time students. □

## Australian art rocks *Homo sapiens*

**Sydney.** Australian archaeologists claimed last week to have found rock art 76,000 years old and stone tools at least 116,000 years old, presenting a direct challenge to accepted theories of the emergence of modern *Homo sapiens* from Africa just 100,000 years ago, and migration by boat to Australia 60,000 years ago (the age of the oldest rock art previously found there). The dating of stone monoliths carved with small circles, and red ochre pigment and stone artefacts found in nearby sediment, has yet to be confirmed. If true, the site in the Northern Territory could indicate either an earlier arrival of *H. sapiens*, or the presence of a 'primitive' predecessor, *H. erectus*, and that the latter was more capable than previously believed. □

## Nicotine research 'destroyed'

**Washington.** Philip Morris, the leading US cigarette manufacturer, sent sensitive scientific research to be destroyed at a German research centre, and planned to "bury" adverse information about nicotine, suggests material that has just been filed with a Minnesota court. Minnesota is among 15 states that are suing tobacco makers over the costs of treating poor citizens for tobacco-related illnesses.

State lawyers have filed a motion intended to force the company to produce all its papers relating to destruction of documents. The motion cites an unsigned, handwritten note discussing sending documents to Inbifo, the company's research centre in Cologne. The note, obtained from the file of Thomas S. Osden, the company's research director from 1969 to 1984, says that documents "will be destroyed", and "if IMPORTANT [sic] letters or documents have to be sent, please send to home — I will act on them & destroy." □

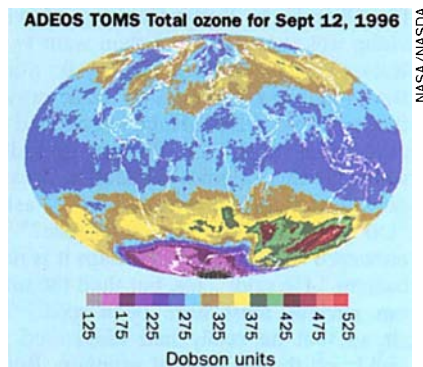
## US conditionally clears RU-486

**Washington.** The US Food and Drug Administration (FDA) has conditionally approved the marketing of the abortion-inducing drug RU-486, long available in Europe but so far barred in the United States for political reasons. The FDA said last week that the drug, known in the United States as mifepristone, is safe and effective.

The announcement clears a substantial hurdle for the drug's sponsor, the Population Council, based in New York, which says that it plans to market the drug by mid-1997. But the FDA has also asked the Population Council for more information on labelling and manufacturing processes. If adequate responses are provided, final approval will follow, the government agency said. □

## Ozone layer monitoring resumes

**London.** Making the ozone hole visible again, this image was obtained on 12 September by NASA's Total Ozone Mapping Spectrometer (TOMS), which is mounted on the Japanese Advance Earth Observing Satellite (ADEOS). The spectrometer has resumed the daily monitoring of the Earth's ozone layer, which was disrupted when a previous instrument stopped working in December 1994. The instrument calculates the amount of ozone present by comparing the level of ultraviolet light emitted by the Sun to that scattered by the Earth's atmosphere. □



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# Hahn's Nobel was well deserved

SIR — I read with interest the report<sup>1</sup> on the internal debates in the Nobel committee that preceded the award of the Nobel prize to Otto Hahn in 1945. I am one of the few surviving witnesses of Hahn's studies leading to the discovery of uranium fission, although I knew nothing at the time about the details.

As a young theoretical physicist, I worked for six months in 1936 with Lise Meitner in her section of the Kaiser-Wilhelm-Institut für Chemie in Berlin-Dahlem, of which Hahn was the director. I then went to the Kaiser-Wilhelm-Institut für Physik, also in Berlin-Dahlem, five minutes' walk away. In the last week of 1938, Hahn telephoned and asked me: "Can you imagine a radium which in every chemical separation does not go with radium but with barium?" I asked: "Do you have such a substance?" He answered "Yes". I said: "Perhaps it is really barium." He said: "Yes, but then the uranium nucleus must have been split." This shows that he really had discovered and identified the splitting of uranium. But, as an empirically working chemist, he felt somewhat uncertain about it and so he asked me, as a theoretical physicist, whether I believed it.

When he wrote to Meitner, who was then in Sweden, he was similarly cautious. From members of his institute, I soon learned that Hahn and Meitner had discussed the problem before she left his institute in the late summer of 1938. Several years previously, Frédéric Joliot in Paris had bombarded uranium with neutrons and produced a substance that he interpreted as a radium isotope. This was very difficult for a physicist such as Meitner to believe. Hahn said: "I must study those 'radium isotopes' of Joliot's." Meitner said: "It isn't worth the effort. It must be an error. It's nonsense." But as soon as she had left the institute, Hahn, with Strassmann, started to repeat Joliot's experiment, and he found the chemical behaviour of barium. Some members of Hahn's institute later told me: "If Lise Meitner had stayed in Berlin, it is quite possible that Hahn would not have started this experiment and so would not have been the one who discovered uranium fission."

When Meitner learned from Hahn's letter what he had found, she, with Otto Frisch, invented a correct model of the process, that is, she confirmed his empirical result by finding a theoretical description of its possibility. It is true that she used a theoretical model of the nucleus invented by Niels Bohr, but it was she and Frisch who first applied it to Hahn's discovery, which they knew about from Hahn's letter before it was published. But she had private contact with Bohr, who confirmed her conclusion.

Thus it was right to give the Nobel prize

for chemistry to Hahn (or perhaps to Hahn and his collaborator Strassmann). Whether Meitner (perhaps with Frisch, that excellent theoretician) should have been given the prize for physics for first confirming the theoretical correctness of Hahn's interpretation of his result is an open question. I think Hahn himself would gladly have accepted that. But the discovery itself was clearly Hahn's.

**Carl Friedrich v. Weizsäcker**

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SIR — Crawford *et al.*<sup>1</sup> deserve credit for their Commentary article in which they shed more light on the circumstances leading to the award in 1945 of the 1944 Nobel prize to Otto Hahn.

However, their statement that "Hahn and his colleagues immediately began writing Meitner out of the discovery of nuclear fission" when, on 6 August 1945, during their detention at Farm Hall, they heard about the atomic bomb on Hiroshima, is a rather one-sided portrayal.

As can be seen from reports by Major T. H. Rittner, their British 'host' at Farm Hall, the detainees became very worried about reports in the next day's newspapers of their alleged work on the atomic bomb in Germany. At Rittner's suggestion, therefore, on 8 August 1945 the Germans drew up a memorandum setting out their side of the story<sup>2</sup>. One of the issues dealt with in the memorandum was the discovery of nuclear fission, which in the newspapers was attributed only to Meitner<sup>3</sup>.

The authors are right to consider the memorandum as showing the Farm Hall detainees "writing Meitner out of the discovery of nuclear fission". But it would have been fair to have stated that the detainees were reacting to newspaper reports that mentioned only Meitner as the discoverer.

**Jan H. J. Oelering**

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1. Crawford, E., Sime, R. L. & Walker, M. *Nature* **382**, 393–395 (1996).

2. *Operation Epsilon: The Farm Hall Transcripts*, 93 (Institute of Physics, Bristol, 1993).

3. Sime, R. L. *Lise Meitner: A Life in Physics*, 323 (University of California Press, 1996).

## No funeral in Berlin

SIR — Alison Abbott recently reported on the poor financial health of the Berlin universities, particularly the "nearly bankrupt Free University" (*Nature* **382**, 486; 1996). The impression was conveyed that demise is imminent and condolences are in order.

It is true that we are suffering from budget cuts more than non-university research institutions. The well-being of the universities is evidently not high on our politicians' priority list. For example, the decision not to house the Institute for Molecular Pharmacology in existing buildings close to the medical school of the Free University but, instead, to erect a new building on the site of a non-university institution, is just another instance of the political myopia that fails to see how the quality of university research will be reflected in the qualifications of the next generation of researchers. Nevertheless, I am happy to report that more than 700 full professors, about 3,000 scientists, and 49,000 students are still working hard and successfully at the Free University of Berlin. So we are suffering because of short-sighted politics but wreaths are premature.

**Klaus Roth**

Free University of Berlin,  
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Germany

## Plants and AIDS

SIR — The News story "Chemistry professor takes over Islamic science organization" (*Nature* **382**, 487; 1996) incorrectly states that a claim has been made that our institute is close to finding a therapy for AIDS. The article was apparently based on a distorted press version of our interesting discovery of four plants that are active against HIV-I. A clarification by us was subsequently published in the national Urdu newspaper *Jang*.

We initiated a collaborative programme with the US National Institutes of Health (NIH) in Bethesda, Maryland, about four years ago to search for plants with anti-cancer and anti-AIDS activities. Plants are collected from remote areas of Pakistan, extracted and the extracts subjected to screening at NIH. As result of this programme, we have discovered four plants with varying degrees of activity against the HIV-I virus.

The active plants are subjected to bioassay-directed fractionation in order to isolate the bioactive constituents. Although we consider the discovery of plants with anti-AIDS activity exciting, we do not claim to be close to finding a "therapy for AIDS". We are at present actively seeking collaboration with hospitals specializing in the treatment of AIDS patients in order to carry out double-blind clinical trials on the phytochemical preparations.

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# Two-bit anion channel really shapes up

David C. Gadsby

**Channels that conduct anions or cations across cell membranes are of immense physiological importance. But what do they look like? A chloride channel turns out to have a double-barrelled structure quite unlike that of cation channels.**

LIKE choosy border guards, ion channels select exactly which kind of ions they will allow to cross cell membranes, and when, and where. They open and close their gates in response to precise orders received in local or broadcast messages. The resulting orchestrated flows of the cations  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{2+}$ , and the anion  $\text{Cl}^-$ , generate nearly all the electrical activity in our brains and hearts, in every nerve and muscle fibre — indeed, in every single cell in our body.

Almost two decades of recording currents flowing through microscopic patches of membrane have probably given us more detailed kinetic information about how ion channels work<sup>1</sup> than for any other class of protein. And yet we don't have much of a clue as to what they look like. Like other integral membrane proteins, they're prohibitively difficult to crystallize — a prerequisite for determining their three-dimensional structure.

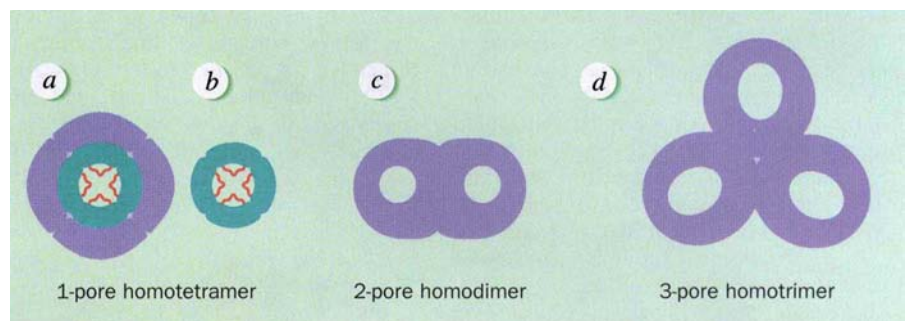
That's the bad news. The good news is that comparisons of wild-type and mutated channels have begun to provide glimpses of their structural features. Much of this work has focused on the reigning stars of the field, voltage-gated cation channels. (All channels, whether selective for a cation or an anion, need energy to swing their gates; common energy sources are the membrane potential and ligand binding.)

Voltage-gated cation channels and their close relatives adopt a pseudo-symmetric tetrameric architecture: a central ion pore lies at the axis of fourfold symmetry and part of its lining is formed by four loops<sup>2</sup>, each contributed by one of the subunits (*a* and *b* in the figure). Now, work from the Miller and Jentsch laboratories (by Middleton *et al.*<sup>3</sup> and Ludewig *et al.*<sup>4</sup>; pages 337 and 340 of this issue) turns the spotlight on the CIC family of anion channels, which are prevalent in skeletal muscle, neurons and epithelia. The new studies establish that the founding member of this family, the CIC-0 channel cloned from the electric organ of the ray *Torpedo*, exists as a homodimer containing two identical and independent, off-axis pores each contributed solely by one or other of the subunits (*c* in the figure). This is a channel structure that has never been encountered before. Indeed, until now, the only structures even remotely resembling it — that is, having a pore in each subunit — were porins, poorly selective, triple-barrelled

channels from bacteria (*d* in the figure).

In terms of structure, function and public relations, anion channels have been the poor cousins of cation channels — with two exceptions. One is the cystic fibrosis transmembrane conductance regulator (CFTR), an epithelial  $\text{Cl}^-$  channel encoded by the gene defective in cystic fibrosis patients. The other is the family of  $\text{Cl}^-$  channels typified by CIC-0, a polypeptide of relative molecular mass 90K that, like CFTR, includes 12 hydrophobic, possibly

What's more, the probabilities of observing the three different levels are accurately predicted by the binomial theorem. And the measured 'dwell' times (which are exponentially distributed) at each of the three levels quantitatively match estimates based on just one of those dwell times and the observed probabilities. This set of results prompted the postulate that CIC-0 might be a double-barrelled channel made up of two identical ion-conduction pores, each with its own independent



Schematic representations (not to scale) of quaternary structures of ion channels viewed from above, emphasizing the multimetric architecture and disposition of the ion-conducting pore. *a*, Voltage-gated cation channel, with a single axial pore at the centre of a homo- or heterotetramer in the case of  $\text{K}^+$  channels, and at the centre of a single polypeptide in a tandem, pseudotetrameric arrangement in the case of  $\text{Na}^+$  or  $\text{Ca}^{2+}$  channels. The four loops (red) are sequences of <20 residues known to protrude into, and define, the pore<sup>2</sup>, and they link two (the fifth and sixth) membrane-spanning regions of each subunit; the first to fourth membrane-spanning regions (purple) include the sequence containing several basic residues that contribute to the voltage sensitivity of gating. *b*, Inwardly rectifying  $\text{K}^+$  channel, with a single axial pore at the centre of a homo- or heterotetramer. *c*, CIC-0  $\text{Cl}^-$  channel, with two off-axis pores, each contributed by one of the subunits of the homodimer. *d*, Porin from the outer membrane of Gram-negative bacteria: here there are three large off-axis pores, each contributed by one of the subunits of the homotrimer.

membrane-spanning segments. Gating of CIC-0 is voltage-sensitive and, as in voltage-dependent cation channels, depolarizing the membrane (reducing the amplitude of the internal negative potential) rapidly opens the channels, while prolonged depolarization slowly inactivates them. But when unitary currents are recorded in single CIC-0 channels, they reveal an extraordinarily rich gating behaviour.

Between electrically silent periods that reflect the inactivated state, the CIC-0 unitary currents vacillate between three equally spaced current levels. The lowest corresponds to the fully closed channel, the middle to a 10-picosiemens channel and the upper to a 20-picosiemens channel. These bursts of unitary currents invariably include both middle and upper levels, never one without the other.

activation gate, but with a slow inactivation gate in common<sup>5</sup>.

Middleton *et al.*<sup>3</sup> and Ludewig *et al.*<sup>4</sup> take this view of CIC-0 channel structure from supposition to certainty. Their experiments exploit hybrid channels composed of wild-type subunits associated with subunits bearing functional tags in the form of point mutations (at the lysine at residue 519 or the serine at residue 123). The mutations alter the ease with which anions negotiate the pore (measured as conductance), and the pore's ability to select among different anions, as well as its pattern of opening and closing. The new measurements show that two pores, with wild-type and mutant signatures, coexist in a hybrid complex (isolated by an affinity tag or by fusing the subunits), and thus that the two pores in a CIC-0 channel

are functionally distinct and independent.

But how many ClC-0 subunits make up one double-barrelled complex? Sedimentation experiments suggested<sup>6</sup> that the purified, active channel is a homodimer of relative molecular mass about 200K. When expressed alone, ClC-0 mutants, such as those with a cysteine at residue 519 and threonine at 123, show a uniformly reduced conductance of both pores in a channel complex; and they recapitulate that same conductance in one of the pores when coexpressed as hybrids with wild-type ClC-0 subunits (also when expressed as fused wild-type-mutant or mutant-wild-type complexes<sup>4</sup>). Given the absence of intermediate conductance levels, it seems that each pore-affecting residue exerts its effect only once per pore, and that ClC-0 channel complexes must therefore be made of just two subunits.

In an elegant, real-time application of the cysteine accessibility method, Middleton *et al.*<sup>3</sup> are able to show that precisely two cysteine-modification events, by a cytoplasmic (but not extracellular) reagent that introduces a positive charge, are necessary and sufficient to convert the low conductance of the cysteine-519 channel to the higher conductance anticipated upon restoring the wild-type charge at residue 519. This both confirms the channel's dimeric structure, and places the lysine at 519 on the cytoplasmic side.

But do both subunits contribute residues to each pore, or does each subunit circumscribe its own pore? Ludewig *et al.*<sup>4</sup> addressed this question by expressing a doubly mutated tandem channel (with glutamic acid at residue 519 in one subunit, and threonine at 123 in the other), incorporating one mutation per subunit, at opposite ends of the linear sequence. The double mutant showed two low-conductance pores as might be expected if each mutation affected just one pore, supporting a model (c in the figure) in which each subunit contributes residues to one pore only. Nonetheless, more detailed analysis of additional double mutants covering the rest of the sequence may be required to settle the matter.

There are, of course, further outstanding issues. If each ClC-0 subunit does make a pore, presumably several parts of the molecule contribute pore-lining residues. But which parts? And what holds the two subunits together? Are the

pores in lone subunits inactivated? Are all ClC family members dimers<sup>3,4,6,7</sup>?

Mutational studies can take us only so far, and full answers must await a high-resolution structure of one of these channels. That entails preparing large quantities of pure protein for growing two- or three-dimensional crystals, and perhaps here too the advantage has passed from cation to anion channels—Miller and colleagues have already exploited a powerful expression system, the *Torpedo* electric organ, to achieve the analytical-scale purification of fully functional ClC-0 channel protein<sup>6</sup>. With progress accelerating in the preparation of protein samples for crystallization, and

so many channelologists lusting for any sign of success, perhaps it won't be too long before we see the first atomic structure of these all-important ion channels.

Until then, the two new studies give us plenty to ponder. They simultaneously close a chapter, by ending debate over the multimeric nature of ClC-0 channels, and open a whole new book on the molecular structure, and mechanisms of gating and permeation, in the family to which they belong. □

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## SUPRAMOLECULAR CHEMISTRY

# The medium is the message

Achim Müller and Christian Beugholt

THE complexity of appearances in nature has forced humans to think about the principles of order and organization. Werner Heisenberg, for instance, contemplated the 'order of reality', and especially its prebiotic origin: "In the

beginning was symmetry; that is certainly more correct than the Democritean thesis, in the beginning was the particle"<sup>1</sup>. Modern science looks for symmetries and symmetry breaking everywhere<sup>2</sup>. So why do we find such high symmetries

in simple and complex molecular systems, and even in viruses? Beissel and co-workers have reported<sup>3</sup> on a principle of symmetry-driven self-assembly deduced from natural products—a principle that they have used to design the formation of highly symmetrical metal clusters.

We could read only a couple of years ago that cluster self-assembly was "... a hit-or-miss affair, with only limited rational planning"<sup>4</sup>. This problem is of general importance, not only for pure academic chemistry but also for the construction of molecular materials and for the purpose of mimicking some of nature's tricks. Beissel *et al.* ask what it is exactly that leads to the high symmetry of non-covalently bonded molecular clusters, like that of virus coats and some special proteins in nature.

Indeed, nature has brought forth molecular clusters of stunning symmetry and beauty. The poliovirus, for instance, has an icosahedral shell with fivefold and threefold symmetry axes (Fig. 1a), con-

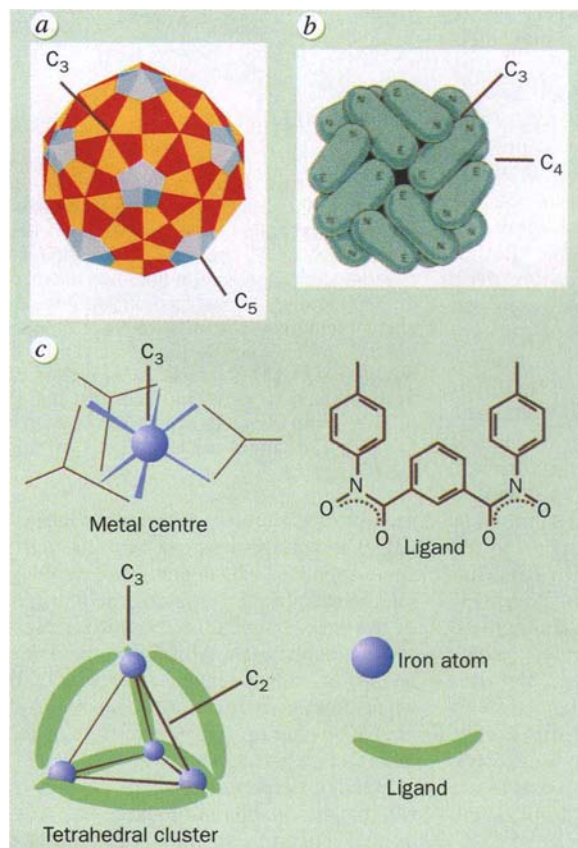


FIG. 1 a, Model of the poliovirus 60-mer (from ref. 5) and b, the apoferritin 24-mer (from ref. 3), showing their symmetry axes. c, The formation of the tetrahedral cluster  $\text{Fe}_4(\text{ligand})_6$  from the designed ingredients: metal centres with their characteristic property of octahedral coordination, and double-bidentate ligands with a twofold axis. This yields a metal tetrahedron with both twofold and threefold symmetry axes.

- Hille, B. *Ionic Channels of Excitable Membranes* (Sinauer, Sunderland, MA, 1992).
- MacKinnon, R. *Neuron* **14**, 889–892 (1995).
- Middleton, R. E., Pheasant, D. J. & Miller, C. *Nature* **383**, 337–340 (1996).
- Ludewig, U., Pusch, M. & Jentsch, T. J. *Nature* **383**, 340–343 (1996).
- Miller, C. & Richard, E. A. in *Chloride Channels and Carriers in Nerve, Muscle, and Glial Cells* (eds Alvarez-Leefmans, F. J. & Russell, J. M.) 383–405 (Plenum, New York, 1990).
- Middleton, R. E., Pheasant, D. J. & Miller, C. *Biochemistry* **33**, 13189–13198 (1994).
- Steinmeyer, K., Lorenz, C., Pusch, M., Koch, M. C. & Jentsch, T. J. *EMBO J.* **13**, 737–743 (1994).



sisting of 60 copies of each of four proteins<sup>5</sup>. Beissel *et al.*<sup>3</sup> discuss the ferritin apoprotein, because when it is denatured its 24 subunits reassemble immediately to re-form the same octahedrally symmetrical protein (Fig. 1b), and not another oligomer.

The formation of this ferritin has been explained by the concept of symmetry-based self-assembly<sup>3</sup>. The noncovalent interactions of the asymmetrical monomers of ferritin are regarded as lock-and-key interactions, to use Emil Fischer's classical terminology. There are two ways that these monomers can interlock, leading to two different local symmetries and elemental make-ups with a fourfold and a threefold axis. As these are incommensurate (they cannot be overlapped), the combination of both types forces the system to adopt a higher symmetry with both types of axis.

Beissel *et al.* went on to mimic this natural construction principle by synthesizing a highly symmetrical metal cluster in the laboratory. The resulting cluster  $\text{Fe}_4(\text{ligand})_6$  is characterized by incommensurate three- and twofold symmetry axes (Fig. 1c). The threefold symmetry axis is induced by using three bidentate (two-toothed) ligand groups to coordinate the metal centre. Each ligand has two bidentate coordinating groups and is designed to have twofold symmetry, so the resulting cluster with six ligands as building blocks and four metal centres must possess tetrahedral symmetry.

Tetrahedral clusters of the same type have been reported before<sup>6</sup>, but not discussed in the context of symmetry-based metal-complex formation. It is important that metal-metal interactions, which would also have a structural influence, are negligible in the present case because of

the length of the ligand. The difference between this and the natural products such as ferritin is that the weak, non-covalent protein-protein subunit interactions are replaced here by highly directional, strong and covalent metal-ligand bonds.

This method may be important not only for mimicking aspects of the organization of special biological products, but also for

both electrons and protons, and can store hydrogen.

The chemistry of these polyoxometalates is unusual in that the degree of polymerization of highly symmetrical monomeric units (platonic solids) can be directed by the solution's acidity<sup>9</sup>. This, together with our ability to generate large transferable fragments in solution, such as the  $\text{Mo}_8$  or  $\text{Mo}_{17}$  units, enables the chemist to create even larger clusters<sup>8,9</sup>. So huge molecular assemblies can be made, leaving the simple microscopic molecular world and entering the mesoscopic one, without going through the difficult task of forming covalent bonds one by one.

The highly symmetrical natural structures we have discussed here are examples of nature's more passive products, such as storage proteins and virus coats. 'Active' materials, such as enzymes responsible for more demanding reaction pathways than storage, are in general more complicated, which means less symmetrical and not as rigid. Nature cunningly produces her more passive, highly symmetrical structures by using many identical repeating units, because that reduces the amount of genetic information necessary. But if we want to understand the processes of nature, we must also understand her pathways to diversity and complexity.

Nature has evolved complex, asymmetrical molecular systems that are perfectly suited to their functions. Complex molecules like proteins are produced in a sequence of steps under dissipative conditions — that is, far from equilibrium. The corresponding challenge for the chemist is to synthesize complex molecules under non-equilibrium conditions using multi-component, one-pot reactions, without separating and purifying each intermediate product, and thus easily produce molecules that are multi-functional and highly active (like enzymes) rather than the symmetrical clusters discussed here<sup>9,11</sup>. In our example, that would mean generating metal-oxide fragments in solution and linking them again without isolation, which is indeed possible with the giant species shown in Fig. 2b.

In contrast, the reaction depicted in Fig. 1c is of a one-step type that leads to the spontaneous assembly of the ingredients, corresponding to the information stored in them<sup>12,13</sup>. In other words, if you make the right sort of bricks, they will do the building on their own. □

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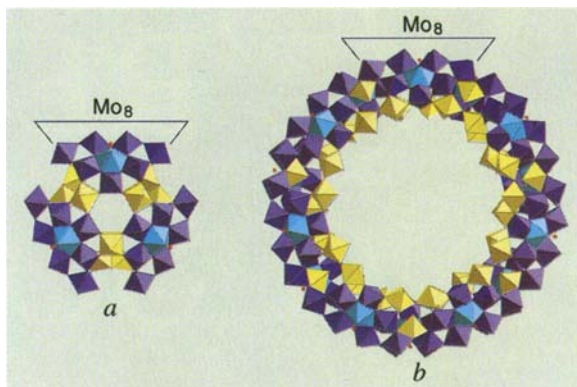


FIG. 2 Polyhedral models of two highly symmetrical clusters. All the polyhedra have oxygen atoms at their vertices and a molybdenum atom in their centres. *a* and *b* contain the same building block,  $\text{Mo}_8$  (blue and turquoise), built up by linking eight of those polyhedra. The  $\text{Mo}_8$  groups are linked in *a* and *b* by bridging groups (yellow polyhedra). The three identical, large, transferable  $\text{Mo}_{17}$  building blocks of *a*, also occurring, differently linked, in other clusters, are built from two  $\text{Mo}_8$  units which are linked by a further molybdenum atom (with this particular view of the model, half of the  $\text{Mo}_{17}$  unit is hidden behind the rest).

designing useful molecular materials. Prefabricated transferable building blocks, such as the ligands in the new synthetic cluster, must be covalently (and therefore non-reversibly) linked<sup>7</sup> to each other to achieve stability.

For example, a large building block containing 17 molybdenum atoms and an adequate number of oxygen atoms can be linked in different ways — by cationic metal centres, for example — to form a highly symmetrical cluster with a central cavity (Fig. 2a). Each of the  $\text{Mo}_{17}$  fragments contains  $\text{Mo}_8$  units as sub-building blocks. These can also be found, differently linked, in several other clusters, for instance in the nano-sized macrocyclic species shown in Fig. 2b, which has 154 molybdenum atoms and a relative molecular mass of about 24,000, and the  $\text{Mo}_8$  units form its skeleton<sup>8,9</sup>. This giant mixed-valency species is water-soluble and a striking deep-blue. Because of the large number of mobile electrons, it could be the first example of a molecular semiconductor. It is also a nice model example of the intensely coloured, mixed-valency, solid-state, metal-oxide bronzes<sup>10</sup>. These materials can be semiconductors or superconductors, and have a wide range of composition — of special interest to materials science are the molybdenum bronzes, which conduct

1. Heisenberg, W. *Collected Works Ser. C, Vol. III: Der Teil und das Ganze* (eds Blum, W., Dürr, H.-P. & Rechenberg, H.) 324–325 (Piper, München, 1985).
2. Mainzer, K. *Symmetries of Nature* (de Gruyter, Berlin, 1996).
3. Beissel, T., Powers, R. E. & Raymond, K. N. *Angew. Chem. Int. Ed. Engl.* **35**, 1084–1086 (1996).
4. Lee, S. C. & Holm, R. H. *Angew. Chem. Int. Ed. Engl.* **29**, 840–856 (1990).
5. Stryer, L. in *Biochemistry* 3rd edn, 866–868 (Freeman, New York, 1988).
6. Saalfrank, R. W. *et al. Angew. Chem. Int. Ed. Engl.* **33**, 1621–1623 (1994).
7. Ball, P. *Nature* **375**, 101–102 (1995).
8. Müller, A. *et al. Angew. Chem. Int. Ed. Engl.* **34**, 2122–2124 (1995).
9. Müller, A., Reuter, H. & Dillinger, S. *Angew. Chem. Int. Ed. Engl.* **34**, 2328–2361 (1995).
10. Wells, A. F. *Structural Inorganic Chemistry* 612–619 (Oxford Univ. Press, 1984).
11. Müller, A. & Mainzer, K. in *From Simplicity to Complexity in Chemistry — and Beyond* (eds Müller, A., Dress, A. & Vögtle, F.) 1–11 (Vieweg, Wiesbaden, 1996).
12. Constable, E. C. *Nature* **362**, 412–413 (1993).
13. Lehn, J.-M. *Supramolecular Chemistry* 143–160 (VCH, Weinheim, 1995).

# The third form of life

Michael W. Gray

How many fundamentally different cell types are there in the living world? Until recently, most biologists would have said two: prokaryotes (cells without a nucleus, such as bacteria) and eukaryotes (cells that have a nucleus, including those of multicellular animals, fungi and plants, as well as unicellular algae and protozoa). But in 1977, Carl Woese and George Fox made a radical, and since controversial, proposal<sup>1</sup> — that a group of supposed bacteria, which Woese and colleagues initially termed archaebacteria and later renamed archaea<sup>2</sup>, are as distinct from true bacteria (eubacteria) as they are from eukaryotes. This conclusion, based on a comparison of ribosomal RNA sequences, led to the concept that organisms divide into, not two, but three primary lineages (domains): Archaea, Bacteria (eubacteria) and Eucarya (eukaryotes)<sup>2</sup> (see figure).

A landmark paper by Bult *et al.*<sup>3</sup>, published in the 23 August issue of *Science*, now gives us the first complete genome sequence from an archaeon. What emerges is a pleasing picture that is neither typically bacterial nor typically eukaryotic, but something in between. Life, it seems, does indeed come in three forms.

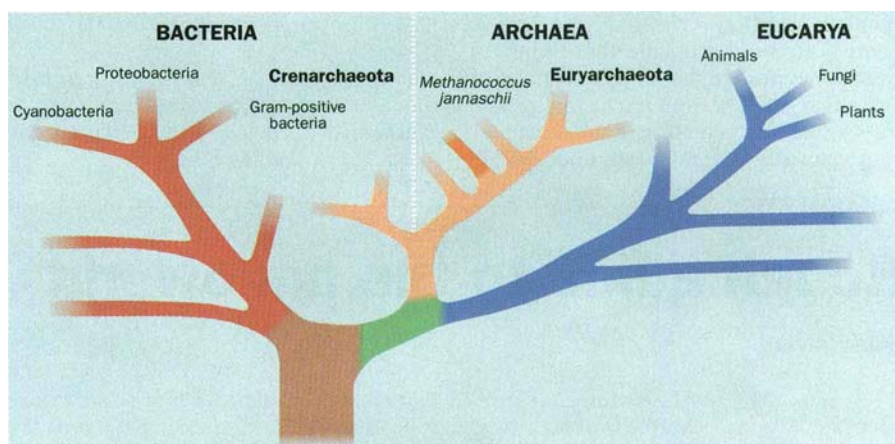
Large-scale genome sequencing has become something of a growth industry, spawning in its wake a new discipline (genomics). Last year, a team from TIGR (The Institute for Genomic Research) reported the first two (eu)bacterial genome sequences, those of *Haemophilus influenzae*<sup>4</sup> and *Mycoplasma genitalium*<sup>5</sup>. Earlier this year, completion of the sequence of the yeast nuclear genome by an international consortium was announced<sup>6</sup>. TIGR's latest effort<sup>3</sup> is the genome sequence of the anaerobic, methanogenic (methane-producing) archaeon, *Methanococcus jannaschii*. With this sequence, complete gene repertoires are now available from representatives of the three proposed domains of life.

Members of the Archaea tend to occupy rather extreme environmental niches, and *M. jannaschii* is no exception. Plucked from the depths (2,600 m) of a Pacific hydrothermal vent by the research submarine *Alvin* in 1982 (ref. 7), *M. jannaschii* is able to grow at pressures above 200 atmospheres and at temperatures as high as 94 °C. Despite its decidedly unconventional lifestyle, *M. jannaschii* has a genome that in physical structure very much resembles that of a typical bacterium — that is, a large, circular chromosome (1.66 million base pairs; 1,682 protein-coding genes), plus two much smaller, circular, extrachromosomal elements (ECEs) of 58,407 base pairs (44 genes) and 16,550 base pairs (12 genes). Clustered genes

(operons) that are coexpressed as polycistronic transcripts (another characteristic bacterial feature) are also evident in the *M. jannaschii* genome, whereas introns, prominent in most eukaryotic nuclear genomes but extremely rare in bacteria, were not reported.

Given these apparent bacterium-like traits, what features distinguish the *M. jannaschii* genome from the two previously sequenced eubacterial genomes? A remarkably large proportion (56 per cent)

of information processing — replication, transcription and translation — are in the main most similar to their eukaryotic counterparts. For example, the transcription machinery in archaea more closely resembles that in eukaryotes than in bacteria<sup>8</sup>. The data of Bult *et al.*<sup>3</sup> emphasize just how close this resemblance is: the *M. jannaschii* genome encodes 12 components of a complex, multi-subunit, DNA-dependent RNA polymerase, seven of which have homologues in eukaryotes but not bacteria. The archaeal genome also contains six genes specifying a eukaryote-like transcription-initiation system. So although archaeal genes may be organized in a bacterial manner (that is, in operons), the



Universal phylogenetic tree (adapted from and rooted as described in ref. 2) showing the three domains of life and illustrating the early divergence of Bacteria, and the sister-group relationship between Archaea and Eucarya. The domain Archaea is split into two kingdoms: Euryarchaeota comprises extreme halophiles (archaea found in high-salt habitats) and methanogens (anaerobic methane-producers such as *M. jannaschii*), whereas Crenarchaeota consists of thermophilic acidophiles (also called eocytes<sup>10</sup>), archaea that grow under conditions of high temperature and high acidity.

of the 1,738 inferred protein-coding genes turned up no convincing match to sequences in the public databases; few of the large ECE and none of the small ECE genes could be identified. In contrast, only 22 per cent of the *H. influenzae* genes could not be matched in a similar search<sup>4</sup>. Some of these unidentified genes undoubtedly specify distinctive features of archaeal biochemistry, but others may represent genes that have yet to be encountered in either bacterial or eukaryotic genomes. Alternatively, they may be so highly diverged in sequence that it is difficult to recognize these as homologues of — that is, sharing a common evolutionary ancestry with — known eubacterial or eukaryotic genes.

What really sets the *M. jannaschii* genome apart is its mix of bacterium-like and eukaryote-like gene sequences. Genes encoding components of central metabolic pathways (including those involved in energy production and nitrogen fixation) are similar between *M. jannaschii* and bacteria, as are certain genes encoding proteins involved in cell division. On the other hand, genes that specify functions

protein machinery expressing them is decidedly eukaryotic in character.

The eukaryotic connection extends to genes for ribosomal proteins, aminoacyl transfer RNA synthetases and protein synthesis factors (although genes for both eukaryote-like and bacterium-like translation initiation factors are present in *M. jannaschii*). Surprisingly, *M. jannaschii* seems to get by with only a single DNA polymerase, a relative of the eukaryotic  $\alpha$ ,  $\delta$  and  $\epsilon$  DNA polymerases. Its replication system evidently uses several homologues of eukaryotic replication initiation factors. Finally, *M. jannaschii* contains five histone genes, two of which are located on the

1. Woese, C. R. & Fox, G. E. *Proc. Natl Acad. Sci. USA* **74**, 5088–5090 (1977).
2. Woese, C. R., Kandler, O. & Wheelis, M. L. *Proc. Natl Acad. Sci. USA* **87**, 4576–4579 (1990).
3. Bult, C. J. *et al. Science* **273**, 1058–1073 (1996).
4. Fleischmann, R. D. *et al. Science* **269**, 496–512 (1995).
5. Fraser, C. M. *et al. Science* **270**, 397–403 (1995).
6. Williams, N. *Science* **272**, 481 (1996).
7. Jones, W. *et al. Arch. Microbiol.* **136**, 254–261 (1983).
8. Klenk, H.-P. & Doolittle, W. F. *Curr. Biol.* **4**, 920–922 (1994).
9. Keeling, P. J., Charlebois, R. L. & Doolittle, W. F. *Curr. Opin. Genet. Dev.* **4**, 816–822 (1994).
10. Rivera, M. C. & Lake, J. A. *Science* **257**, 74–76 (1992).



large ECE. The implied presence of histones suggests that although the *M. jannaschii* gene map looks rather bacterial, the genome itself may actually be organized *in vivo* in a fashion more typical of eukaryotic than bacterial chromosomes.

The data and analysis of Bult *et al.*<sup>3</sup> not only confirm the distinctiveness of the archaea, but support other work over the past decade which indicates that they are specific relatives of the eukaryotes<sup>9</sup> (see figure). Given the insights into the archaeal world provided by the new sequence, additional archaeal genome sequences are eagerly awaited. Woese and colleagues have further divided the domain Archaea into two kingdoms: Euryarchaeota, of which *M. jannaschii* is a member, and Crenarchaeota<sup>2</sup> (see figure). There is continuing debate about whether crenarchaeotes are the specific relatives of the eukaryotes, to the exclusion of the euryarchaeotes<sup>10</sup>. Sequencing of a number of crenarchaeotal genomes is underway in

several laboratories, and the results will no doubt further refine our ideas about evolutionary relationships among the three domains, and especially between Archaea and Eucarya. Considering where *M. jannaschii* was discovered, and the fact that it is hardly a garden-variety microbe, it is striking that so many of its genes are like so many of ours.

As a bonus, Bult *et al.*<sup>3</sup> have given us, for the first time, the complete genetic complement of an autotroph: an organism that is able to synthesize all its required biomolecules from inorganic compounds. It is at once sobering and exhilarating to realize that among the 1,738 *M. jannaschii* genes is all the genetic information that is both necessary and sufficient for completely independent cellular life. □

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## MATERIALS SCIENCE

# Rough guide to the nanoworld

Paul Calvert

WHY do we want nanotechnology? "Because it's not there" is the answer given by Thomas Bein and Galen Stucky in their preface to the August issue of *Chemistry of Materials*, which contains a collection of reviews and papers discussing nano-structured materials, and provides a perspective on the clever things we can already do. The techniques described will provide the basis for the future production of nanostructured mechanical and electrical components.

As particles become much smaller than one micrometre, the attractive forces between them become larger in comparison to external mechanical forces. Hence, it gets progressively more difficult to grind finer powder, to break up aggregates and to keep dispersions uniform and unagglomerated. At the nanometre scale, particles become mainly surface, and familiar materials may acquire new electronic or optical properties. As mechanical forces cease to have much effect, manipulations must be made by changes in surface chemistry. In the context of mechanical properties, 'nanometre scale' tends to mean less than 100 nm, whereas for electronic properties it means less than 10 nm.

Nanoparticles have been experimented on for a long time. Faraday produced ruby-coloured solutions of gold particles that have been stable for more than a hundred years. Commercial fumed silica, made by gas-phase reaction, has a ten-nanometre particle size and is so fluffy that a litre weighs only a few grams. Many ceramic and metal-alloy systems can be manipulated so

that nanometre particles precipitate from a solid matrix. For instance, photosensitive sunglasses and many coloured glasses contain metal nanoparticles.

So materials containing nanometre-sized grains have many interesting properties, but they do not promise to change the world. What might change the world is our growing ability to use surface interactions to make shaped particles for mechanical reinforcement, and to construct clusters, lines and arrays of nanoparticles to be

used in electronic and mechanical devices. Both hinge on the need for a better understanding of what happens at disordered interfaces between unrelated materials. We have some insight into what happens when one semiconductor is grown on another, but know little about what happens when a metal is deposited on a polymer or a protein on glass.

When an existing structure defines the shape of a growing particle, it seems reasonable to think of it as a mould. But just as rock can be split by ice, any growing mineral crystal should be able to push its way through a confining polymer. So the 'mould' wall is not a hard barrier — it must exert its control by subtler surface effects or through the ability of reagents to permeate only one phase. A very soft structure can thus control the form of a hard particle growing within it.

In the synthesis of organic-inorganic hybrid materials<sup>1</sup>, the object is to combine the toughness of plastic with the hardness of glass. In fact, we have largely combined the brittleness of glass with the softness of plastic (although we can make good scratch-resistant coatings for plastic). In these hybrids, the silica particles that form within the polymer matrix are essentially spherical — so perhaps non-spherical particles would produce stiff and tough composites. One way to introduce such particles may be by exfoliating clay plates into polymers<sup>2</sup>.

Solid-platelet-filled polymer composites show a much more rapid change in stiffness with mineral content than do polymers filled with spherical nanoparticles, but they are still not as tough as we would like. Block copolymers can now be manipulated to give rod- and plate-like two-phase structures<sup>3</sup>, and it is shown that these rods can be used as nanometre-sized

moulds for mineralization<sup>4</sup>, but a high mineral content has not yet been achieved, so they are not strongly reinforcing. Several groups report in the journal how elongated pores or soft rods in a two-phase polymer can be used as moulds to grow shaped inorganic particles *in situ*<sup>5,6</sup>. That is important because it is most unlikely that we will be able to disperse rods or sheets into a matrix by direct mixing. Like a bag of nails, the rods would lie at awkward angles and not pack well.

Silicon integrated circuit technology is already sub-micrometre, so it is a form of nanotechnology, but the properties of silicon limit what can be done. It has two obvious shortcomings. First, we want to incorporate sen-



Gold colour: these alumina membranes have cylindrical pores into which gold nanowires have been electrochemically deposited. The wire diameters go from 150 nm (top row) to 20 nm (bottom row), and aspect ratios increase from left to right (from ref. 5). Changing colour shows the changing electronic structure with particle size and shape.

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sor arrays on chips, but silicon is not sensitive to chemicals or to the small environmental changes detected by our own skin senses. To get around those deficiencies we will need to grow localized sub-micrometre structures of softer materials on silicon surfaces. But organic structures will not stand up to the rigours of evaporation, baking and etching that go with lithography, so we need to form the materials on a surface template using some kind of solution chemistry. Second, silicon technology is essentially two-dimensional: a charge-coupled device is an excellent model for a retina, but there is really no way of growing the equivalent of an eye. For that, we need guided sub-micrometre deposition in three dimensions.

Various groups give examples of the formation of films of nanoparticles at interfaces, or on surfaces that have been treated to induce mineralization<sup>7-9</sup>. Nanoparticles have been manipulated by scanning tunnelling microscopy<sup>10</sup>, and although doing this one particle at a time may be impossibly slow, it might be possible to develop a multi-particle transfer system analogous to the contact printing technique described by Whitesides and colleagues<sup>11</sup> for patterning reagents on surfaces. Once we can form patterns, we can deposit many layers to form interconnected three-dimensional structures.

This volume isn't a description of nanotechnology, because that does not exist yet. It is a toolbox for nanotechnology. Now we have to decide what to make. For materials, that is a difficult problem, because a large improvement in properties would be needed to justify the extra manufacturing effort needed, compared with existing polymers, metals or ceramics. But a tough ceramic, or a mouldable material with the properties of carbon-fibre composite, or a completely impermeable, hard coating for plastics would all be excellent justification for the higher cost. For devices — such as the electronic equivalents of a nose, a fingertip or a complete insect — and for membranes or catalysts, the need is much clearer. □

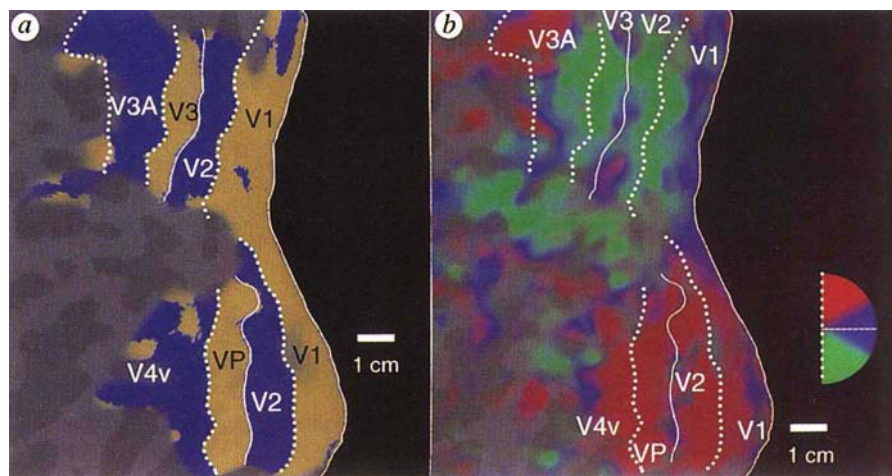
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## Stimulating brain but not mind

C. Koch and R. B. H. Tootell

ONE might think that scientists trying to study consciousness would have a wealth of relevant facts and experiments to work with. Yet given the elusive nature of the phenomenon, some of the most provocative findings have come from a handful of clinical studies and psychophysical experiments indicating a clear divorce between behaviour and certain aspects of visual awareness or consciousness. This sparse database now receives a significant addi-

patches of gratings to either side of the original grating — which remained exactly as before — removed the grating from visibility; it was now 'masked'. Subjectively, one still sees 'something' at the location of the original grating, but one is unable to make out its orientation (subjects cannot reliably indicate grating orientation, even when given unlimited viewing time). Yet despite this inability to 'see' the adapting stimulus, the after-effect was as strong and



a, Topography of human visual cortices, based on data from functional magnetic-resonance imaging (fMRI). The visual stimuli produced fMRI activity at corresponding mapped regions of cortex. The field sign of this retinotopic mapping (that is, neighbouring locations in the image map onto neighbouring locations in cortex) is either consistent with (blue) or mirror-symmetric to (yellow) the visual field geometry seen by the subject<sup>9</sup>. The retinotopy reverses field sign at the anatomical borders indicated, with the representations of vertical and horizontal meridian indicated by dotted and solid lines, respectively. The data, generated by one of us (R. B. H. T.) in collaboration with A. M. Dale and M. I. Sereno, were obtained from the left hemisphere of one normal subject. The grey matter was unfolded, with regions corresponding to gyri or sulci coded as lighter or darker grey, respectively. Superior is up and inferior is down. b, Representation of polar angle retinotopy in the same subject shown in a. When the slowly rotating stimulus wedge was in the upper visual field, it produced activity in the inferior visual cortical areas (red, see inset). Conversely, lower-field stimulation produced activity in superior cortex (green). If human superior and inferior representations of the third visual area are functionally and anatomically distinct areas (V3 and VP), as described in the macaque, area VP may be preferentially involved in the tasks described by He *et al.*<sup>1</sup>, compared with area V3.

tion from the elegant experiment reported by He *et al.* on page 334 of this issue<sup>1</sup>.

The basic design is so simple that it is bound to be widely replicated. It is based on a common visual after-effect (related in kind to the well-known waterfall illusion or motion after-effect). If a subject stares for a fraction of a minute at a horizontal grating and then looks at a faint test grating to decide whether it is oriented vertically or horizontally, the subject's sensitivity for detecting a horizontal grating will be reduced. This adaptation is orientation specific — the sensitivity for vertical gratings is almost unchanged — and disappears quickly.

He *et al.* projected a single patch of grating onto a computer screen. It was clearly visible and their subjects showed the predictable orientation-selective adaptation effect. Adding one or more similar

as specific to the orientation of the 'invisible' grating as when the grating was visible.

What this clearly shows, foreshadowed by earlier experiments<sup>2</sup>, is that visual awareness must occur at a higher stage in the visual hierarchy than orientation-specific adaptation. This after-effect is thought to be mediated by oriented neurons in primary visual cortex (V1) and beyond, implying that the neurons that mediate visual awareness must be located past this stage. The same 'crowding' methodology could be exploited to hide visual targets with other features (colour, motion and texture, for instance) from conscious vision, and test to what extent they lead to measurable behavioural effects.

Further experiments of He *et al.* establish that making the target invisible by adding adjacent gratings works better in the upper field of view than in the lower

1. Wen, J. & Wilkes, G. L. *Chem. Mater.* **8**, 1667–1681 (1996).
2. Krishnamoorti, R., Vaia, R. A. & Giannelis, E. P. *Chem. Mater.* **8**, 1728–1734 (1996).
3. Honeker, C. C. & Thomas, E. L. *Chem. Mater.* **8**, 1702–1714 (1996).
4. Kane, R. S., Cohen, R. E. & Silbey, R. *Chem. Mater.* **8**, 1919–1924 (1996).
5. Martin, C. R. *Chem. Mater.* **8**, 1739–1746 (1996).
6. Walsh, D. & Mann, S. *Chem. Mater.* **8**, 1944–1953 (1996).
7. Hoekstra, K. J. & Bein, T. *Chem. Mater.* **8**, 1865–1870 (1996).
8. Fendler, J. H. *Chem. Mater.* **8**, 1616–1624 (1996).
9. Calvert, P. & Rieke, P. *Chem. Mater.* **8**, 1715–1727 (1996).
10. Shinya, N., Konno, T. & Egashira, M. *SPIE Proc.* **2722**, 36–44 (1996).
11. Xia, Y., Kim, E. & Whitesides, G. M. *J. Electrochem. Soc.* **143**, 1070–1079 (1996).

visual field. They observe the same asymmetry in two, attentional demanding, detection and tracking (search) tasks. Given the absence of any profound difference in visual resolution between the upper and the lower visual field, the authors conclude that attentional resources are more finely deployed in the lower than in the upper field.

What is the likely site for this asymmetry? The lower visual field maps onto cortical areas above (superior to) the depth of the calcarine fissure in the back of the brain, while the upper visual field maps onto the expanse of cortex below (inferior to) this demarcation (see figure *a*). No gross anatomical asymmetries have been reported in either monkey or human upper and lower V1 or V2. So the new findings provide support for the idea, proposed by Crick and one of us (C. K.)<sup>3</sup>, that the neural correlate of awareness is not expressed in V1 neurons but must lie beyond. Whether or not this has anything to do with the fact that V1 has no direct projection to the frontal lobe, as originally surmised, remains completely open.

The first stage where substantial asymmetries in the upper and lower visual fields have been reported is in the third visual area. In the macaque, the areas anterior to the dorsal and ventral parts of V2 are so different from each other that they have been treated as distinct<sup>4,5</sup> and termed V3 proper (the dorsal or superior part) and VP (ventral posterior area; see figure *b*). In particular, V3 receives a direct input from V1, whereas VP does not. These areas also differ functionally: the incidence of direction-selective cells in V3 is higher than it is in VP. For colour, the reverse is true. If there are similar differences in the equivalent areas in humans — as suggested by data from functional magnetic-resonance imaging<sup>6</sup> — they would provide a convenient marker for evaluating the extent to which neurons in the V3/VP complex mediate the observed attentional asymmetries (see figure *b*).

The conclusions of He and his colleagues also bear on the issue of the 'grain of visual awareness', that is, the visual resolution that is accessible to attention. The reader should try, for instance, to count heads in a crowd scene while fixating the centre of the image. Even accounting for the roughly inverse decline of visual acuity with retinal eccentricity, we have great difficulty with this task, possibly because neurons at higher stages that code for the

different elements of the image inhibit one another, leading to reduced firing rates and reduced performance.

A wonderful feature of science is that if there is a clearly defined problem then somebody will usually invent a method to address it. Thus, in the past two years, we have seen the introduction of several psychophysical methods that bypass visual awareness, yet still cause behavioural effects. Two recent examples are very fine gratings that cannot be perceived yet can still cause an orientation-dependent after-effect<sup>7</sup>, and induced blindsight in which subjects can 'guess' the location of a figure that remains hidden from awareness<sup>8</sup> (although blindsight using dichoptic displays, so that each eye can be stimulated

separately, has proven to be very sensitive to the exact evaluation procedure; M. J. Morgan, J. A. Solomon and A. J. S. Mason, personal communication). Now we have the procedure described by He *et al.* It is possible that these techniques, combined with functional imaging<sup>9</sup>, will enable us to pin down the elusive neural correlate of consciousness. □

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## OCEANOGRAPHY

# Microbial ferrous wheel

David L. Kirchman

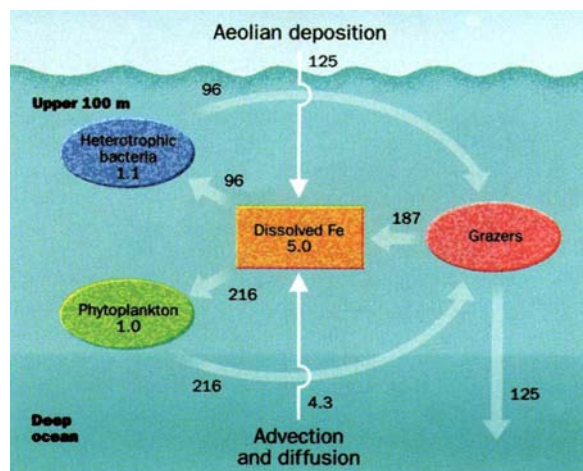
It has been nearly ten years since John Martin and colleagues suggested that iron limits primary production in some oceanic regimes, a hypothesis that turned oceanography upside down at the time. Since then we have come to know much about how iron regulates phytoplankton growth rates and how it interacts with other processes, especially grazing, to control the 'biological pump' — that is, the export of materials (most importantly carbon) to the deep ocean.

Although much has been learned, a report by Tortell *et al.*, which appears on page 330 of this issue<sup>1</sup>, illustrates how far we have to go. In experiments carried out in the subarctic Pacific, Tortell *et al.* found large amounts of iron in heterotrophic bacteria and high uptake rates, spokes of a 'microbial ferrous wheel' (as coined by Farooq Azam, although 'feric wheel' would, chemically, be more correct). It was in the subarctic Pacific that Martin and Fitzwater<sup>2</sup> first demonstrated iron limitation, which explained in part why phytoplankton growth is not more luxuriant here despite abundant plant nutrients.

Oceanographers did not have to trouble with heterotrophic bacteria in thinking about iron limitation, but cyanobacteria have been very much part of the iron story. These photosynthetic microbes, which are functionally akin to eukaryotic phytoplankton, can contribute much to primary production in the open ocean. Heterotrophic bacteria, in contrast, live on dis-

solved organic matter released by many processes and are the first component of the microbial loop which can mineralize a large fraction of primary production. Oceanographers knew about the microbial loop, but not the microbial ferrous wheel.

Of course, it is not news that heterotrophic bacteria contain iron. These microbes have a few of the same iron-



An iron budget for the subarctic Pacific. Rates are in nmol Fe m<sup>-2</sup> d<sup>-1</sup> and standing stocks in  $\mu\text{mol Fe m}^{-2}$ , all taken from the paper by Tortell *et al.*<sup>1</sup>. Grazing rates were assumed to balance iron uptake rates. Regeneration and export rates of iron were assumed to follow analogous rates of the nitrogen cycle (the *f* ratio) for this region<sup>3</sup>. Brand<sup>10</sup> summarized rates of aeolian deposition and inputs from the deep ocean.

bearing components found in phytoplankton, most notably nitrate reductase, the enzyme required for nitrate use. The largest ferric lode in heterotrophic bacteria, however, is in the electron transport chain of oxidative phosphorylation, which a bacterium uses to obtain energy during oxidation of organic carbon to CO<sub>2</sub>. Identifying why a microbe needs iron is easy; the hard part is to estimate quantitatively

1. He, S., Cavanagh, P. & Intriligator, J. *Nature* **383**, 334–337 (1996).
2. Blake, R. & Fox, R. *Nature* **249**, 488–490 (1974).
3. Crick, F. C. & Koch, C. *Nature* **375**, 121–123 (1995).
4. Burkhalter, A. & Van Essen, D. C. *J. Neurosci.* **6**, 2327–2351 (1986).
5. Newsome, W. T., Maunsell, J. H. R. & Van Essen, D. C. *J. Comp. Neurol.* **252**, 139–153 (1986).
6. Tootell, R. B. H. *et al.* *Neuroimage* **3**, 3358A (1996).
7. He, S., Smallman, H. S. & MacLeod, D. I. A. *Invest. Ophthalm. Vis. Sci. Suppl.* **36**, 2010 (1995).
8. Kolb, F. C. & Braun, J. *Nature* **377**, 336–338 (1995).
9. Sereno, M. I. *et al.* *Science* **268**, 889–893 (1995).

the amount of cellular iron, or as oceanographers like to express it, the Fe:C ratio. This ratio is critical in understanding how iron affects carbon cycling, the biological pump and the role of oceanic biota in global climate.

The Fe:C ratios found by Tortell *et al.* will be a surprise to many. In iron-deficient cultures and in the subarctic Pacific, Fe:C ratios in heterotrophic bacteria are twice those in eukaryotic phytoplankton, a difference that is not predictable even after reviewing the biochemistry of aerobic heterotrophy and photosynthesis. Cell size probably has something to do with the difference, as does the energetic cost of heterotrophic bacteria living in organic-poor waters such as the open oceans (more on this later). Further work on Fe:C ratios in cyanobacteria and in heterotrophic eukaryotic protozoa may help sort out whether being a prokaryote or a heterotroph is more decisive in determining the high iron content of heterotrophic bacteria.

Although it is unclear why Fe:C ratios in bacteria and phytoplankton differ, the data clearly indicate that in the subarctic Pacific the heterotrophic bacterial assemblage contains more iron than either eukaryotic phytoplankton or cyanobacterial communities. That observation may not surprise microbial ecologists familiar with work showing that the standing stocks of bacteria and phytoplankton are roughly equal in the open oceans<sup>3</sup>. In spite of their known ecological importance, however, bacteria have been completely ignored in discussions of how iron controls phytoplankton growth; the main battle has been over the role of grazing by zooplankton.

Tortell *et al.* now decisively add bacteria to the fray. What may be more important than the amount of iron is the rate at which heterotrophic bacteria take it up. These rates are much more difficult to come by, but fortunately several different methods turn up roughly the same answer. The take-home message is that heterotrophic bacteria account for 20–45 per cent of total iron uptake in the subarctic Pacific, suggesting that bacteria are competing with phytoplankton for this potentially limiting micronutrient.

As well as having a higher surface-area-to-volume ratio, bacteria can excrete ligands (siderophores) in order to complex and ultimately assimilate dissolved iron, an uptake mechanism unknown to eukaryotic phytoplankton. It may be more than coincidental that the binding constants of these bacterial siderophores are similar to those of dissolved iron complexes in seawater<sup>4</sup>.

Completing the microbial ferrous wheel is iron regeneration, the input of iron back to the dissolved pool by grazing and viral lysis. Like the nitrogen cycle, iron regeneration supports much primary production in many parts of the world's oceans<sup>5</sup> (see figure). Unlike nitrogen and other plant nutrients, the form of released

iron may differ greatly depending on the prey (bacteria or phytoplankton) and the mode of regeneration (viral lysis or grazing by protists and multicellular organisms). The end result is that this microbial ferrous wheel — the uptake, storage and ultimate release of iron from heterotrophic bacteria — may greatly affect iron availability and thus primary production in waters such as the subarctic Pacific.

Might heterotrophic bacteria be limited by iron like some phytoplankton? A recent report<sup>6</sup> says so, but most microbial ecologists think that bacterial growth rates are limited by organic carbon in open oceans. Tortell *et al.* suggest a more subtle limitation, involving both carbon and iron deficiency. They observed that the growth efficiency of several bacterial strains was lower under iron limitation, probably because of deleterious effects on the iron-rich electron-transport system. If so, to maintain growth rates bacteria would require more organic material as iron concentrations decrease. As a result, bacterioplankton could exhibit both carbon and iron deficiencies. The potential effect of iron on the bacterial growth efficiency is quite important; this efficiency indicates whether carbon flows through bacteria either to CO<sub>2</sub> or to higher trophic levels via bacterivores.

A model that incorporates both grazing and iron limitation for understanding what controls primary production is really not much more complicated than the iron-ruled ocean first proposed by Martin and colleagues. A microbial ferrous wheel, however, complicates our simple models, and we will need further work before we see how everything meshes together. An important opportunity may be part of the Southern Ocean programme of the US Joint Global Ocean Flux Study, which went to sea a few weeks ago. The story of iron limitation began in the subarctic Pacific and an epiphany was realized with work in the equatorial Pacific<sup>7</sup>, but its climax may be in the Southern Ocean. All three areas have relatively low phytoplankton growth in spite of high concentrations of major plant nutrients. But it is the vast Southern Ocean that matters most for the biological pump and its role in global climate<sup>8</sup>. □

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1. Tortell, P. D., Maldonado, M. T. & Price, N. M. *Nature* **383**, 330–332 (1996).
2. Martin, J. H. & Fitzwater, S. E. *Nature* **331**, 341–343 (1988).
3. Cho, B. C. & Azam, F. *Nature* **332**, 441–443 (1988).
4. Rue, E. L. & Bruland, K. W. *Mar. Chem.* **50**, 117–138 (1995).
5. Hutchins, D. A., DiTullio, R. G. & Bruland, K. W. *Limnol. Oceanogr.* **38**, 1242–1255 (1993).
6. Pakulski, J. D. *et al.* *Nature* **383**, 133–134 (1996).
7. Landry, M. R. *et al.* *Limnol. Oceanogr.* (in the press).
8. Sarmiento, J. L. & Orr, J. C. *Limnol. Oceanogr.* **36**, 1928–1950 (1991).
9. Wheeler, P. A. *Prog. Oceanogr.* **32**, 137–161 (1993).
10. Brand, L. E. *Limnol. Oceanogr.* **36**, 1756–1771 (1991).

## Astromagnetics

How did the first stars form? The accepted theory is that a large volume of very dilute hydrogen gas slowly collapsed into stars by gravitational contraction. But such masses would warm up as they grew denser, generating pressure which would oppose the contraction. Only by radiating their heat away could the proto-stars contract further. Yet hydrogen is almost perfectly transparent, and therefore hardly radiates at all. Daedalus has been musing on the problem.

The early Universe, he says, had a magnetic field. One theory even claims that the fields of the stars are 'fossil' remnants of it, trapped and concentrated in the stars as they condensed. Now, materials with unpaired electrons are paramagnetic, and are attracted into a magnetic field. Interstellar hydrogen is an extreme case: it consists of single atoms with one electron each, so all its electrons are unpaired. Daedalus calculates that a field of 80 T could by itself pump cold monatomic hydrogen at one atom per cubic metre right up to the billions of atmospheres of a stellar interior. So in forming the first stars, he says, magnetism came to the aid of gravity.

Magnetic fields have tricks unknown to mere gravitational ones. They are always multipolar, for a start, and can be dragged around by moving magnetic material. The Sun heats its corona much hotter than its surface by exporting energy as hydromagnetic waves. Daedalus reckons the early stars did the same thing. The turbulence and spin of their contraction allowed them to radiate energy, not thermally, but magnetically.

These deductions have practical uses. One planned satellite will carry a huge neodymium–boron magnet, to look for anti-matter cosmic rays by their opposite deflection in its field. Daedalus expects it to concentrate interplanetary monatomic hydrogen as well. It could then form the basis of a deep-space rocket motor which would attract this gas as fuel. It would catalyse its conversion to dihydrogen — the most energetic chemical reaction known. The resulting hot exhaust would develop both thermal and magnetic thrust. Dihydrogen is diamagnetic, and would be repelled away from the motor.

Daedalus has even been dreaming of a satellite so intensely magnetized that it could hold an atmosphere of molecular oxygen, also a paramagnetic gas. It would thus form a tiny planet for astronauts to walk on, needing no space-suits but wearing iron-soled boots for adhesion. The oxygen atmosphere would even give them a faintly blue sky. Sadly, the field required exceeds the capacity of modern magnets.

David Jones



# Thermoregulating lotus flowers

**SIR** — The sacred lotus, *Nelumbo nucifera* Gaertn. (Nelumbonaceae; see cover), not only produces a significant amount of heat during the sequence of flowering, but also regulates its temperature with an unexpected precision. We report that in air temperatures varying between 10 and 30 °C, the flowers remain between 30 and 35 °C throughout a 2–4-day period. Thermal stability is achieved by increasing the rate of heat production in proportion to the decrease in ambient temperature, a pattern identical to that in homeothermic birds and mammals. The homeothermic episode begins before petal opening and continues through the period of stigma receptivity. It may enhance and stabilize flower development and perhaps reward insect pollinators with a warm, equable environment.

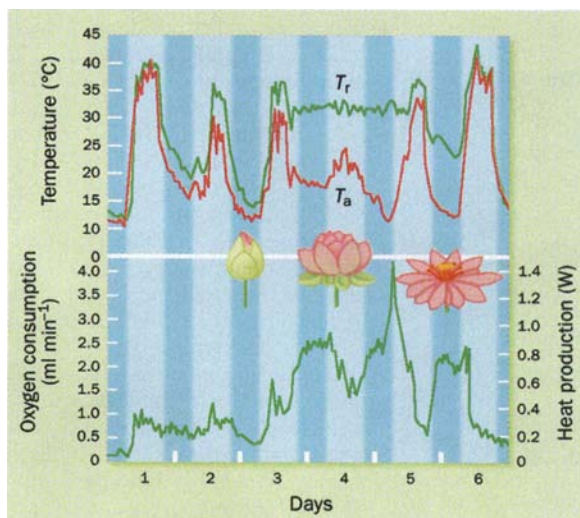
Heat production by flowers and cones is known to occur in many species of the aroid family<sup>1</sup>, and in a few water lilies<sup>2</sup>, palms<sup>3</sup>, and cycads<sup>4</sup>. Although records of heat production are common, observations of temperature stability are limited to two species of aroid: *Philodendron selloum*<sup>5</sup> and *Symplocarpus foetidus*<sup>6</sup>. Temperature regulation in *Nelumbo* has apparently evolved independently. *Nelumbo* flowers are protogynous and have a variable sequence of anthesis that favours outcrossing via insect pollinators<sup>7</sup>. A conical receptacle that contains several carpels forms the bottom of an otherwise empty floral chamber before the petals open (see figure). Stamens grow around the receptacle and are initially pressed close to it by the petals, but are released when the petals open fully.

We measured receptacle and ambient air temperatures and oxygen-consumption rates continuously in 19 flowers in the Adelaide Botanic Gardens. Early buds were at ambient temperature, but a small unregulated temperature rise appeared in closed flowers a few days before the main episode of heating (see figure). Then a 'homeothermic' period of high, regulated temperatures began in many cases 1 or 2 days before the flower opened, and continued for 2–4 days in association with presumed female receptivity (moist stigmas). Temperatures and oxygen consumption started to decline after the petals opened fully and pollen was shed.

During the homeothermic period, receptacle temperature ( $T_r$ ) is regulated in a narrow range (30–35 °C) at ambient air temperatures ( $T_a$ ) of between 10 and 30 °C. At  $T_a = 10$  °C,  $T_r = 30$  °C and at

$T_a = 30$  °C,  $T_r = 35$  °C. At  $T_a > 30$  °C,  $T_r$  rises and remains a few degrees higher than  $T_a$ . Meanwhile, oxygen-consumption rate (hence heat production) is inversely related to  $T_a$  (see figure). Maximum heat production is about 1 W at a  $T_a$  of 10 °C.

Temperature regulation in *Nelumbo* apparently occurs at the cellular level by a steep, temperature-induced, reversible inactivation of the heat-producing meta-



An example of temperatures of the receptacle ( $T_r$ ) and ambient air ( $T_a$ ), and rates of oxygen consumption throughout a complete episode of thermogenesis in the sacred lotus. Stages of flowering and light cycle are indicated. Note the constancy of  $T_r$  and the reciprocal changes in  $T_a$  and oxygen-consumption rate between days 3 and 5. Temperatures in and around the flowers were measured with thermocouples and oxygen-consumption rate was measured with a flow-through respirometry system. Oxygen consumption is converted to heat production assuming 21.1 J per ml of oxygen<sup>12</sup>. The flowers were shielded from direct sunlight with an umbrella.

bolic pathways, as has been observed in thermoregulating *Philodendron*<sup>8</sup>. Briefly, when the flower enters its homeothermic period, it heats itself to at least 30 °C. Above this temperature, further increases in  $T_r$  markedly decrease the rate of heat production. Temperature equilibrium is reached when the rate of heat production equals the rate of heat loss. At low  $T_a$ , therefore, the rate of heat loss is high and  $T_r$  stabilizes near 30 °C. At higher  $T_a$ , heat loss decreases, so  $T_r$  rises slightly and reduces the rate of heat production until equilibrium is reached. Conversely, as  $T_a$  drops, heat loss increases, the flower cools slightly and increases heat production. The homeothermic period therefore relies on small changes in flower temperature within the region just above 30 °C. Regulation depends directly on  $T_r$  and indirectly on  $T_a$ , but apparently not on time of day or light cycle. Temperature stability over 2–4 days indicates complete reversibility of the responses.

Laboratory measurements on cut flowers show that the receptacle is responsible for about half of the heat production, and the petals and stamens about a quarter each. A respiratory quotient of 1.0 indicates that carbohydrate, probably starch, is oxidized in all three tissues. Starch is present in *Nelumbo* staminal appendages, which, until now, have been thought to be the major site of heat production<sup>2,7,9</sup>.

Common explanations for heat production in flowers are that it enhances the evaporation of floral scent that attracts insects<sup>1,7</sup>, that it protects flowers from cold<sup>10</sup>, or that it is a reward for pollinators, particularly endothermic flying insects<sup>8</sup>. Schneider and Buchanan<sup>7</sup>, among several others, have concluded that the floral structure and pattern of anthesis of *Nelumbo* were appropriate for beetle pollination. They found that beetles were important pollinators in several Texas populations, but bees appeared important in another. Beetles that were trapped overnight fed and copulated in the floral chamber and were released unharmed the next morning to carry the pollen away. One function of thermal stability, therefore, may be a direct energetic reward to pollinators. The environment in the floral chamber would maintain a high body temperature for the insect and promote not only feeding, digestion and reproductive behaviour, but also suitable body temperatures for flight. Many insects, including beetles and bees, require thoracic temperatures above 30 °C to initiate flight<sup>11</sup>, and the flower could be directly preparing them for departure, thus eliminating their requirement for endogenous heat production.

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1. Meeuse, B. J. D. & Raskin, I. *Sex. Plant Reprod.* **1**, 3–15 (1988).
2. Skubatz, H., Williamson, P. S., Schneider, E. L. & Meeuse, B. J. D. *J. Exp. Bot.* **41**, 1335–1339 (1990).
3. Schroeder, C. A. *Principes* **22**, 26–29 (1978).
4. Skubatz, H., Tang, W. & Meeuse, B. J. D. *J. Exp. Bot.* **44**, 489–492 (1993).
5. Nagy, K. A., Odell, D. K. & Seymour, R. S. *Science* **178**, 1195–1197 (1972).
6. Knutson, R. M. *Science* **186**, 746–747 (1974).
7. Schneider, E. L. & Buchanan, J. D. *Am. J. Bot.* **67**, 182–193 (1980).
8. Seymour, R. S., Bartholomew, G. A. & Barnhart, M. C. *Planta* **157**, 336–343 (1983).
9. Comi, C. *Nuovo Giorn. Bot. Ital.* **46**, 600–610 (1939).
10. Knutson, R. M. *Am. Mid. Nat.* **88**, 251–254 (1972).
11. Heinrich, B. *The Hot-Blooded Insects. Strategies and Mechanisms of Thermoregulation* (Harvard Univ. Press, Cambridge, 1993).
12. Wieser, W. *Bioenergetik* (Thieme, Stuttgart, 1986).

## Oestrogen and mental state

**SIR** — Oestrogen is thought to exert powerful effects on mood, mental state and behaviour in women. Here we show that an acute surge of oestrogen in the female rat induces a significant increase in the density of 5-hydroxytryptamine<sub>2A</sub> (5-HT<sub>2A</sub>) receptors in higher centres of the forebrain, suggesting that this may be a key mechanism in the psychotropic effect of oestrogen. The role of oestrogen in affective disorders (depression and mania) is suggested by the fact that menopausal and postnatal depression are associated with a massive drop in plasma

oestrogen concentrations, and oestrogen has been reported to be effective in the treatment of depression in women<sup>1–5</sup>. Differences in the age of onset and symptoms of schizophrenia in women compared with men has also implicated oestrogen in schizophrenia<sup>6–8</sup>.

Affective disorders have long been attributed to defective serotonin transmission. Psychopharmacological data suggest that defective serotonin transmission may also be responsible, at least in part, for schizophrenia in that 'atypical' antipsychotics such as clozapine and risperidone,

which are highly effective in the treatment of schizophrenia, bind with much greater affinity to 5-HT<sub>2A</sub> than to dopamine receptors<sup>9–11</sup>. Oestrogen, in its positive-feedback mode for triggering the ovulatory gonadotrophin surge, stimulates a threefold increase in the amount of 5-HT<sub>2A</sub>-receptor messenger RNA in the dorsal raphe nucleus of the female rat<sup>12</sup>; indeed, a single pulse of oestrogen significantly increases the density of 5-HT<sub>2A</sub> binding sites in cerebral cortex and the nucleus accumbens<sup>13</sup>. These findings were based on autoradiography using as ligand [<sup>3</sup>H]ketanserin in the presence of prazosin

(to block binding to  $\alpha_1$ -adrenoreceptors). Because of their potential importance it was crucial to verify these results with a highly selective 5-HT<sub>2A</sub> ligand, RP62203 (ref. 14).

We now report that oestrogen significantly increases the binding of [<sup>3</sup>H]RP62203 in anterior frontal, anterior cingulate and the primary olfactory cortex and in the nucleus accumbens (see figure and table), essential brain regions for cognition, emotion, mental state and mood, as well as neuroendocrine control<sup>13</sup>. As with [<sup>3</sup>H]ketanserin and other 5-HT<sub>2A</sub> ligands<sup>13</sup>, in frontal and cingulate cortex the density of [<sup>3</sup>H]RP62203 binding sites was greatest in laminae IV and Va.

The concordance between the present [<sup>3</sup>H]RP62203 effects and our previous<sup>13</sup> findings using [<sup>3</sup>H]ketanserin<sup>13</sup> provides compelling evidence that the acute effects of oestrogen on mood and mental state are mediated at least in part by an increase in density of 5-HT<sub>2A</sub> receptors. Our experimental model mimics the changes in plasma oestrogen concentrations that occur during the human menstrual cycle, immediately after parturition and at the time of the climacteric (menopause onset). Because oestrogen also affects expression of the gene for the serotonin transporter<sup>14</sup>, the target for highly effective antidepressants such as fluoxetine (Prozac), further investigations are required to determine whether the effect of oestrogen on the 5-HT<sub>2A</sub> receptor is more pertinent to schizophrenia or to affective disorders.

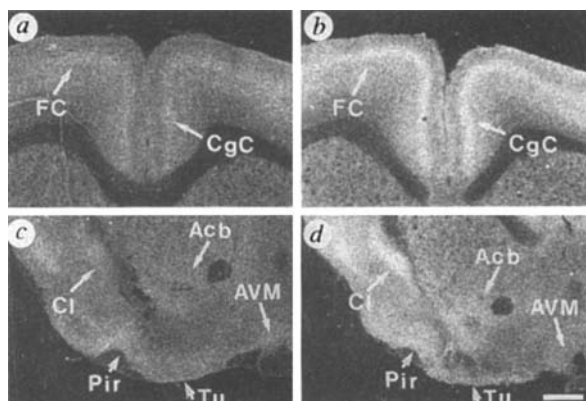
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Film autoradiographs of coronal sections of the forebrain showing [<sup>3</sup>H]RP62203 binding in brain regions of an oil-treated (a, c) compared with an oestradiol benzoate-treated (b, d) ovariectomized rat. FC, frontal cortex; CgC, cingulate cortex; Cl, claustrum; Acb, nucleus accumbens; Pir, piriform cortex; Tu, olfactory tubercle; AVM, anteroventral periventricular nucleus region and medial preoptic area combined. Dark-field photography; scale bar, 1 mm.

[<sup>3</sup>H]RP62203 BINDING IN BRAIN REGIONS OF OVARIECTOMIZED RATS TREATED WITH OESTRADIOL BENZOATE (OB) OR VEHICLE (OIL)

| Brain region              | Oil-treated  | OB-treated   | Per cent increase caused by OB | Paired t test (P) | Wilcoxon signed rank test (P) |
|---------------------------|--------------|--------------|--------------------------------|-------------------|-------------------------------|
| Anterior frontal cortex   | 159.3 ± 17.4 | 200.7 ± 17.5 | 26                             | 0.0010            | 0.0156                        |
| Anterior cingulate cortex | 164.8 ± 14.6 | 194.4 ± 16.3 | 18                             | 0.0041            | 0.0156                        |
| Piriform cortex           | 118.5 ± 14.5 | 148.0 ± 16.8 | 25                             | 0.0101            | 0.0156                        |
| Olfactory tubercle        | 93.9 ± 8.9   | 108.2 ± 12.7 | 15                             | 0.0340            | 0.0312                        |
| Nucleus accumbens         | 113.0 ± 10.7 | 133.5 ± 13.5 | 18                             | 0.0332            | 0.0156                        |
| Clastrum                  | 160.7 ± 17.0 | 168.5 ± 19.0 | 5                              | 0.1452            | 0.0781                        |
| AVPv region               | 89.1 ± 5.6   | 95.6 ± 6.5   | 7                              | 0.1232            | 0.0781                        |
| MPOA adjacent to AVPv     | 88.7 ± 5.9   | 92.4 ± 6.6   | 4                              | 0.2282            | 0.0547                        |

The above means (±s.e.m.) were calculated from measurements in three sections per brain region per rat, and six rats per treatment group. Measurements in frontal and cingulate cortex were made on laminae IV and Va where there was the greatest density of [<sup>3</sup>H]RP62203 binding. The experimental method and quantitative and statistical analysis of autoradiograms were as described in ref. 13 and the binding of [<sup>3</sup>H]RP62203 determined as described in ref. 15. AVPv, anteroventral periventricular nucleus; MPOA, medial preoptic area.

- Dean, C. & Kendell, R. E. *Br. J. Psychiatry* **139**, 128–133 (1981).
- Studd, J. & Zamblera, D. *Focus Depress.* **2**, 6–9 (1994).
- Klaiber, E. L., Broverman, D. M., Vogel, W. & Kobayashi, Y. *Arch. Gen. Psychiatry* **36**, 550–554 (1979).
- Montgomery, J. C. et al. *Lancet* **i**, 297–299 (1987).
- Gregoire, A. J. P., Kumar, R., Everitt, B., Henderson, A. F. & Studd, J. W. W. *Lancet* **347**, 930–933 (1996).
- Di Paolo, T. *Rev. Neurosci.* **5**, 27–42 (1994).
- Häfner, H. et al. *Psychol. Med.* **23**, 925–940 (1993).
- Lewis, S. Br. *J. Psychiatry* **161**, 445–450 (1992).
- Meltzer, H. Y. in *Psychopharmacology: The Fourth Generation of Progress* (eds Bloom, F. E. & Kupfer, D. J.) 1277–1286 (Raven, New York, 1995).
- Nordström, A.-L. et al. *Am. J. Psychiatry* **152**, 1444–1449 (1995).
- Farde, L. et al. *J. Clin. Psychopharmacol.* **15**, S19–S23 (1995).
- Sumner, B. E. H. & Fink, G. *Mol. Cell. Neurosci.* **4**, 83–92 (1993).
- Sumner, B. E. H. & Fink, G. *J. Steroid Biochem. Mol. Biol.* **54**, 15–20 (1995).
- McQueen, J. K., Wilson, H., Dow, R. C. & Fink, G. *J. Physiol. (Lond.)* **495**, 114P (1996).
- Malgoures, C., Flamand, F. & Doble, A. *Eur. J. Pharmacol.* **233**, 37–45 (1993).

### Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. Priority will be given to letters of fewer than 500 words and five references.

## Battle of the sexes

**SIR** — Sexual conflict can result in rapid evolutionary change. Rice<sup>1</sup> found that male and female fruitflies, *Drosophila melanogaster*, are continually forced to counteract adaptations in the other sex to maintain their fitness (see also the discussion of this paper in News and Views<sup>2</sup>). Rice concluded that "intersexual coevolution... [can] contribute substantially to genetic divergence among physically isolated or semi-isolated populations". But does sexual conflict invariably operate as an 'engine of speciation'? There is at least one case where it appears to have the opposite effect.

The guppy, *Poecilia reticulata*, is a small poeciliid fish, native to Trinidad and northeast South America. It is an ovoviparous species with a promiscuous mating system. Guppy populations in Trinidad show marked differentiation and have become a classic example of evolution in action<sup>3</sup>. Natural selection, in the guise of predators, accounts for much of the variation; experiments have revealed rapid population divergence following a shift in predation regime. For example, heritable changes in male colour patterns<sup>4</sup>, life-history traits<sup>5</sup> and antipredator behaviour<sup>6</sup> occur within a few years (from 10 to 100 generations) of a reduction in predator pressure.

It is not only natural selection that drives evolution in this species; sexual selection is also a significant diversifying agent with the potential to reinforce population differences that predators have generated. Female guppies exert choice and base their mating preferences on individually variable male colour patterns. Female preferences and male coloration co-vary across populations<sup>7</sup>. Houde<sup>8</sup> has uncovered a genetic correlation between

male coloration and female choice which may facilitate speciation<sup>9</sup>. Once mating has occurred, females can store sperm and fertilize several broods without further contact with a male. A single female can even found a viable population<sup>10</sup>. The genetic drift resulting from such severe founder events may further magnify population differences.

Although many of the elements required for rapid evolution are present, guppy populations, in Trinidad at least, do not appear to be speciating<sup>3</sup>. Populations that have been separate for about 2 million generations<sup>11,12</sup> will interbreed if given the opportunity to do so<sup>13,14</sup>. How might this paradox be resolved?

It seems that while many aspects of the behaviour and biology of female guppies hasten differentiation, a number of male traits hinder it. Wild female guppies are subject to a barrage of sneaky mating attempts, receiving, on average, one per minute<sup>15</sup>. The relative success rate of sneaky matings is unknown. However, the

high incidence of sneaky matings is such that only a few need to be successful to undermine female choice. Female preference may be further compromised by competitive mating among males<sup>13,16</sup>. In addition, male guppies are much more likely to emigrate than females (D. N. Reznick, personal communication). The net result is that the geographical scale of gene flow is large relative to the scale of the selection regime<sup>3</sup> and reproductive isolation, which is the precursor of speciation, has little opportunity to develop. Of course, sexual conflict is not the only factor that shapes the destiny of guppy populations, but it must play a significant, if previously unrecognized, role. The battle of the sexes does indeed have profound evolutionary consequences.

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## Na<sup>+</sup> channel subunits and Ig domains

**SIR** — Voltage-gated sodium channels isolated from brain cells are heterotrimeric complexes composed of a central, pore-forming  $\alpha$ -subunit, of relative molecular mass 260,000 ( $M_r$  260K), and two auxiliary subunits,  $\beta_1$  (36K) and  $\beta_2$  (33K) (ref. 1). The  $\alpha$ -subunit is a polytopic transmembrane glycoprotein, whereas the  $\beta_1$  and  $\beta_2$  subunits are single membrane-spanning glycoproteins. The  $\beta_1$  and  $\beta_2$  subunits are unique among ion-channel subunits studied to date in that their extracellular domains contain immunoglobulin-like motifs, similar to those found in many cell-adhesion molecules<sup>2</sup>. In addition, the  $\beta_2$  subunit has a segment with striking amino-acid sequence similarity to the cell-adhesion molecule contactin<sup>2</sup>. Because nearly all of the immunoglobulin-like motifs so far discovered interact with extracellular protein ligands<sup>3</sup>, the  $\beta_1$  and  $\beta_2$  sodium-channel subunits probably also serve this function. As sodium channels are highly localized in neurons and are immobilized in the neuronal plasma membrane<sup>4</sup>, interaction of the  $\beta_1$  and  $\beta_2$  subunits with extracellular proteins could function in the targeting, membrane insertion and immobilization of sodium channels.

Previous analyses of the structures of immunoglobulin-like motifs have defined

three structural sets: the C1-, C2- and V-sets<sup>3</sup>. According to these previous criteria, most cell-adhesion molecules in the nervous system, including contactin, NCAM and myelin-associated glycoprotein, are included in the C2-set of immunoglobulin-like motifs<sup>3</sup>. But based on analysis of the many more immunoglobulin-like motifs that have become available since the initial classification, a revised structural classification and revised consensus sequences for the C2-set and the V-set were proposed in a recent review<sup>5</sup>. In this new structural classification, the neural cell-adhesion molecules contactin, NCAM, myelin-associated glycoprotein, DCC, DM-GRASP, telencephalin and Thy-1 are all in the V-set rather than the C2-set.

The consensus sequence derived from analysis of the sequences of these neural cell-adhesion molecules and other V-set immunoglobulin-like motifs is illustrated in the figure, together with the corresponding residues from the  $\beta_1$  and  $\beta_2$  subunits. This comparison shows that the immunoglobulin-like motifs of the  $\beta_1$  and  $\beta_2$  subunits belong to the V-set according to this new consensus sequence. Of eight positions in the consensus having predicted hydrophobic amino acids,  $\beta_1$  contains seven and  $\beta_2$  six. Both subunits contain a conserved tyrosine in the appropriate

1. Rice, W. R. *Nature* **381**, 232–234 (1996).
2. Chapman, T. & Partridge, L. *Nature* **381**, 189–190 (1996).
3. Endler, J. A. *Trends Ecol. Evol.* **10**, 22–29 (1995).
4. Endler, J. A. *Evolution* **34**, 76–91 (1980).
5. Reznick, D. N., Bryga, H. & Endler, J. A. *Nature* **346**, 357–359 (1990).
6. Magurran, A. E., Seghers, B. H., Carvalho, G. R. & Shaw, P. W. *Proc. R. Soc. Lond. B* **248**, 117–122 (1992).
7. Houde, A. E. & Endler, J. A. *Science* **248**, 1405–1408 (1990).
8. Houde, A. E. *Proc. R. Soc. Lond. B* **256**, 125–130 (1994).
9. Lande, R. *Proc. Natl Acad. Sci. USA* **78**, 3721–3725 (1981).
10. Carvalho, G. R., Shaw, P. W., Hauser, L., Seghers, B. H. & Magurran, A. E. *Biol. J. Linn. Soc.* **57**, 219–234 (1996).
11. Fajen, A. & Breden, F. *Evolution* **46**, 1457–1465 (1992).
12. Magurran, A. E., Seghers, B. H., Shaw, P. W. & Carvalho, G. R. *Adv. Study Behav.* **24**, 155–202 (1995).
13. Magurran, A. E., Paxton, C. G. M., Seghers, B. H., Shaw, P. W. & Carvalho, G. R. *Behaviour* **133**, 503–517 (1996).
14. Endler, J. A. & Houde, A. E. *Evolution* **49**, 456–468 (1995).
15. Magurran, A. E. & Seghers, B. H. *Proc. R. Soc. Lond. B* **255**, 31–36 (1994).
16. Kodric-Brown, A. *Anim. Behav.* **44**, 165–167 (1992).

|           |                                                         |
|-----------|---------------------------------------------------------|
| V-like    | Gxx*x*x*C ... *xW ... +* ... Lx*xx*xxxDx#xYxC ... *x*x* |
| $\beta_2$ | LPCTFNSC ... LNW ... KI ... VTLLKNVQLEDEGIYNC ... IYLOV |
| $\beta_1$ | FKILCISC ... TEW ... KI ... IFITNVTYNHSGDYEC ... IHLEV  |

Consensus alignment of immunoglobulin V-like domains. Asterisks, hydrophobic amino acids; crosses, basic amino acids; hashes, Gly, Ala, Asp; X, any amino acid.



position. Basic residues and glycines are present in both subunits where predicted. A critical tryptophan is located 14 residues from the initial cysteine of the immunoglobulin-like motif in  $\beta 1$  and 12 residues from it in  $\beta 2$ , in agreement with the consensus. Therefore, the neural cell-adhesion molecules and the sodium channel  $\beta 1$  and  $\beta 2$  subunits all contain structurally related V-like immunoglobulin motifs. These new structural comparisons strengthen the implication that the immunoglobulin-like motifs of the  $\beta 1$  and  $\beta 2$  subunits act like neural cell-adhesion molecules in interacting with extracellular protein ligands. Identification of the extracellular ligands for these subunits

may shed light on the mechanisms of localization and immobilization of sodium channels in neurons.

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1. Isom, L. L., DeJongh, K. S. & Catterall, W. A. *Neuron* **12**, 1183–1194 (1994).
2. Isom, L. L. *et al.* *Cell* **83**, 433–442 (1995).
3. Williams, A. F. & Barclay, A. N. *Annu. Rev. Immunol.* **6**, 381–405 (1988).
4. Joe, E. H. & Angelides, K. J. *J. Neurosci.* **13**, 2993–3005 (1993).
5. Vaughn, D. E. & Bjorkman, P. J. *Neuron* **16**, 261–273 (1996).

## Alternative hydrogen cloud models

**SIR** — The ratio of deuterium (D) to hydrogen (H) in the early Universe is one of the fundamental checks of our understanding of the Big Bang. First observations gave an unexpectedly high D/H ratio for primeval, high-redshift hydrogen clouds<sup>1–5</sup> and suggest a very low baryon content for the Universe. A recent paper by Tytler *et al.*<sup>6</sup>, which finds a D/H ratio in a new high-redshift cloud less than one-fifth that of earlier estimates, has provoked considerable controversy over the deuterium abundances at high redshift. Here I show that it is possible to construct alternative cloud models that are both consistent with the published data of Tytler *et al.*<sup>6</sup> and give D/H ratios that are 3–4 times higher than their value. A final choice between the high D/H values and the low value favoured by Tytler *et al.*<sup>6</sup> will require an accurate determination of the

residual continuum level near the hydrogen series limit. The very high precision quoted by Tytler *et al.*<sup>6</sup> for their D/H estimate results from the neglect of alternative models.

Only a small fraction of the high-redshift hydrogen clouds have enough column density and a simple enough velocity structure to produce observable deuterium lines, and also have sufficiently low column density that there is only moderate optical depth in the hydrogen continuum. For such systems it is possible to determine the hydrogen column densities in ways that are insensitive to modelling parameters. But as the cosmic abundance of deuterium is low<sup>7</sup>, the deuterium lines are faint and their strength can be confused by blending with one of the many unrelated hydrogen lines that densely populate high-redshift spectra. Because

any contamination would increase the apparent deuterium abundance, it is safest to quote these D/H ratios only as upper limits. The new Tytler *et al.*<sup>6</sup> cloud has several times the hydrogen column density previously found in systems used for D/H estimates<sup>1–5</sup>. As the accompanying deuterium line is also relatively strong, the probability that the deuterium line is significantly contaminated by unrelated hydrogen absorption is low. But the hydrogen spectrum is saturated and this greatly increases the sensitivity of the derived hydrogen column density determination to modelling assumptions. At issue is whether one can find a model that simultaneously satisfies the constraints of the new spectrum while

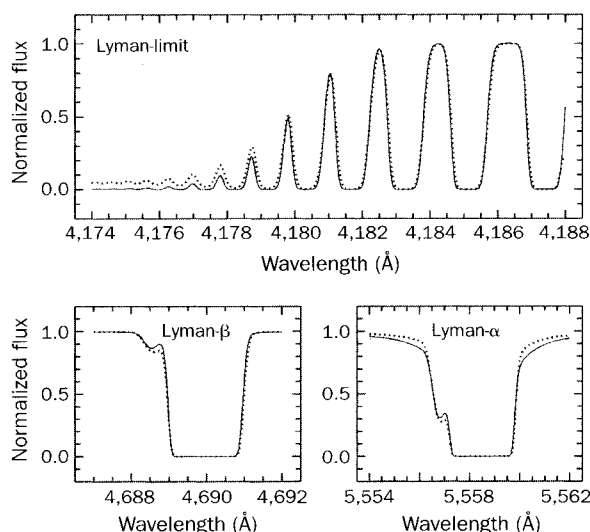
giving a D/H ratio consistent with earlier estimates<sup>1–5</sup>.

When absorption lines are blended and saturated, great freedom is available in the choice of fitting parameters. As all these choices should be treated as free parameters, the set of possible solutions that are consistent with the data is large. By increasing the number of velocity components, the absorption can be more evenly distributed over the line profile and less column density is required to produce deep, broad lines. The remaining free parameters can then be suitably adjusted to match the line wings. The figure shows the Tytler *et al.*<sup>6</sup> fit (solid line) together with one of the numerous alternative models (dotted line). This alternative model reduces the hydrogen column density by using three instead of two discrete cloud velocities and results in a D/H ratio that is three times that quoted by Tytler *et al.*<sup>6</sup>.

There are two important differences between the old fit and the new model: at a wavelength of 4,176.6 Å (Ly22) the residual intensity has increased from ~0% to ~2.5%, and at Ly $\alpha$  the damping wings are much weaker than before. These two differences are a consequence of the reduced hydrogen column density and are unavoidable in all low-hydrogen models. For any model to be acceptable, such differences must not be so great as to be inconsistent with the data. In the published data the Ly $\alpha$  damping wings are blended with deuterium and other unrelated lines. Consequently, their weakness in the new model is less of a potential problem than is the increased residual intensity at wavelengths below Ly22. Further decreasing the hydrogen column density by an additional factor of 1.5 increases the residual intensity at Ly22 to ~8.6% of the local continuum. Although the spectrum may not permit this large a departure from the Tytler *et al.*<sup>6</sup> fit, a close inspection of their Fig. 2 does suggest that for Ly14 to Ly20 the zero level of the fit has been systematically set above data by a few per cent of the local continuum. If further observations/analyses do show that the residual continuum below 4,177 Å is as high as 10%, models that are both consistent with the data and have D/H > ~10<sup>-4</sup> can be constructed.

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Comparison of the two-cloud fit of Tytler *et al.*<sup>6</sup> (solid line) and the alternative three-component model discussed here (dotted line). The cloud parameters for the new model are:  $N_H = 1, 1.2, 2.6 (\times 10^{17} \text{ cm}^{-2})$ ;  $b = 16, 18, 23 (\text{km s}^{-1})$  and redshift  $z = 3.5721101, 3.5722401, 3.5724301$ . The D/H ratio for the new model is  $6.3 \times 10^{-5}$ , compared to  $2.3 (\pm 0.3 \pm 0.3) \times 10^{-5}$  for the Tytler *et al.*<sup>6</sup> fit.

1. Songaila, A., Cowie, L. L., Hogan, C. J. & Rugers, M. *Nature* **368**, 599–603 (1994).
2. Carswell, R. F. *et al.* *Mon. Not. R. Astron. Soc.* **268**, L1–L4 (1994).
3. Rugers, M. & Hogan, C. J. *Astrophys. J.* **459**, L1–L4 (1996).
4. Carswell, R. F. *et al.* *Mon. Not. R. Astron. Soc.* **278**, 506–518 (1996).
5. Wampler, E. J. *et al.* *Astron. Astrophys.* (in the press).
6. Tytler, D., Fan, X.-M. & Burles, S. *Nature* **381**, 207–209 (1996).
7. Schramm, D. N. & Turner, M. S. *Nature* **381**, 193–194 (1996).

# Geological tales of old

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**Thinking About the Earth: A History of Ideas in Geology.** By David Oldroyd. *Athlone*: 1996. Pp. 410. £50. Published in the United States by Harvard University Press, \$45.

POOR Helen must have been flummoxed. It was 16 October 1843 and she was walking in to Dublin from Dunsink Observatory alongside her husband, Sir William Rowan Hamilton, the Astronomer Royal of Ireland. Suddenly, as they crossed the Royal Canal by Broome Bridge, Sir William produced a pocket-knife, rushed to the parapet of the bridge, and scratched thereupon the fundamental formula for quaternions:  $i^2=j^2=h^2=ijh=-1$ . In his own words, "an electric circuit seemed to close; and a spark flashed forth". Today a plaque marks the site.

I know exactly how Hamilton felt. I had an identical experience. In July 1984 (I think it was the 26th of the month) I was walking from Dun Laoghaire to Dublin, when, just outside Blackrock College, my own mental circuit closed and I knew that I had the fundamental formula for the Earth sciences. I rushed to the wall of the college but found that I lacked a pocket-knife with which to record my moment of *eureka*. In any case, my wall was a tougher proposition than Hamilton's; mine was granite and his was limestone. As a result, my formula has until now remained entirely unrecorded. The formula is:  $rm^n + fl^n + ho^{l^0} + pt = eh$ , where  $r$  is rocks,  $m$  is minerals,  $f$  is fossils,  $l$  is landforms,  $ho$  is human observers,  $pt$  is prevailing theory and  $eh$  is Earth history. To the superscripts, incidentally, I attach little significance. Hamilton had some so I thought I would introduce a few just for cosmetic effect.

Owing to my tardiness in bringing the formula to the notice of the scientific world, David Oldroyd has naturally remained oblivious of its existence. The formula is nevertheless fundamental to this, his latest book. Over the millennia, human observers could scarcely avoid encounter with the rocks, minerals, fossils and landforms amidst which we eke out our lives. To those objects we have ascribed a temporal dimension. We see them as a legacy from a geohistorical past. We hold the human mind to be capable of using them as windows into the past, just as Sherlock Holmes at the Abbey Grange used a bent poker, a torn bell-rope and three wineglasses in brilliant reconstruction of the gruesome events of the previous evening.

Thus a distinctive sequence of sedimentary rocks becomes the basis for an eighteenth-century account of precipitation within some primordial liquid, or a

twentieth-century account of plate divergence. A cluster of minerals in one age serves to inspire a tale of vegetative growth under celestial influence, and in another age a tale of hydrothermal activity during orogenesis. Ammonites at one moment serve to generate romances about serpents massacred through saintly invocation, while at another they become evidence of mass extinction related to asteroid impact and an iridium anomaly at the Cretaceous-Tertiary boundary. Landforms such as eskers are by seventeenth-century observers seen as sandbanks shaped in Noah's flood, whereas the modern observer holds them to be the casts of the subglacial streams active during the Pleistocene ice age. Every geologist is tattooed with the aphorism: "The present is the key to the past". Perhaps the inscription should be modified slightly to read: "The present is the key to a past".

To ascribe to the Earth a past is to build a theatre. In that theatre we stage the geohistorical dramas inspired within us by the terrestrial objects of our present world. Once our impresarios were mired, and they insisted upon declaiming their own prologue taken from the Old Testament.

In those days, a mere three hundred years distant, our theatre was in consequence tiny and the action extremely cramped. Now our impresarios inhabit radiometric laboratories, and in their print-outs they offer us use of a theatre vast beyond human imagining. In that immense auditorium we mount the remarkable multi-authored geospectacular that is our version of the history of the global surface. And there we have the essence of Oldroyd's book.

The book is concerned on the one hand with the geohistorical dramas that were written in the past, which received public performance, which achieved popularity, but which then fell from favour. On the other hand, it is concerned with the writing of that modern version of the geohistorical drama that is today captivating audiences throughout the world. Oldroyd's subtitle tells it all. This is just a history of geological ideas. If I may commandeer one of the author's favourite verbs — 'eschews' — he here eschews any claim to the parentage of a comprehensive history of geology, and he confines himself to the Western world.

Oldroyd's panorama is nonetheless huge. As the chapters unfold, he conducts us from the creation myths of the ancient world, through the ambitious seventeenth-century theories of the Earth, and into the increasing sophistication of the nineteenth century with its national geological surveys, its seismological explorations of the Earth's interior, and its new-found beliefs in periodic desiccation, episodic glaciation and titanic denudation. Finally, we arrive at the



Rock of ages — the Bentonite Hills in the Capitol Reef National Park, Utah, are made up of deposits laid down 140 million years ago. The picture — taken more recently — comes from the lavishly illustrated *Hot Spots: America's Volcanic Landscape* by the photographers Diane Cook and Len Jenshel. Bulfinch, \$50.

present through a nicely concocted account of the evolution of plate-tectonic theory, and the whole delectable edifice is topped off — as might be expected of a chef from ‘down under’ — with a summary of the history of Warren Carey’s ideas on the subject of an expanding Earth. Those familiar with Oldroyd’s previous literary offerings might hope to find this new work well laced with philosophical liqueur. I can assure them that their palate will not here suffer disappointment.

Our author has carried his researches into some remote literary corners, and his 38-page bibliography must prove of inestimable value to future scholars. He expresses the hope that those who devour his pages will find them easier to read than he found them to write. I appreciate the problems that he faced. He had set for himself a gargantuan task. But he can rest content. His hope is fulfilled. I found the book easy and enjoyable reading, although I did crave for a splash of colour in some of the many illustrations.

Oldroyd once thought of attempting a biography of Sir Archibald Geikie, the only geologist ever to have served in the presidential chair of the Royal Society (1908–13). As I closed Oldroyd’s book, it was Geikie who entered my mind. In 1896, exactly one hundred years ago, the president of Johns Hopkins University in Baltimore, Maryland, invited Geikie to inaugurate its series of George Huntington Williams memorial lectures. Geikie accepted, but one aspect of his response must surely have evoked surprise in Baltimore. Instead of devoting his discourses to some theme lifted from the core of the geological science, he proposed to lead his audiences out to graze upon pastures within the history of science. The published version of those lectures — Geikie’s *The Founders of Geology* — proved to be highly influential. For half a century it remained the dominant work in its field.

The publication of Oldroyd’s book is an important event. It is the most broadly significant English-language addition to the literature of the history of the Earth sciences since the appearance of the first edition of Geikie’s work in 1897. Oldroyd’s words deserve to be read — his conclusions deserve to be pondered — by all those desirous of insight into the nature of the geohistorical drama that, over the centuries, we have compiled both for human edification and for human entertainment. □

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■ *Genesis and Geology* by Charles Gillespie, first published in 1951, has just been reissued in paperback with a new foreword by Nicolaas Rupke. Harvard University Press, \$17.95, £11.95.

## A weird world

Tony Leggett

**Where Does the Weirdness Go? Why Quantum Mechanics Is Strange, but Not as Strange as You Think.** By David Lindley. *Basic Books*: 1996. Pp. 251. \$24.

THE scope and purpose of David Lindley’s book is well conveyed by its title. It is quite simply an account, in entirely non-mathematical terms, of what is odd and counter-intuitive about the quantum-mechanical view of the world at the level of electrons and photons, and why this same account, when extrapolated to the everyday world, does not (in the author’s opinion) lead to any real difficulties or paradoxes.

These two questions take up, roughly speaking, the first three-quarters, and last quarter of the book, respectively. In the first three-quarters, Lindley for the most part does an excellent job; the writing is clear, incisive and down to earth, and succeeds remarkably well in explaining difficult concepts without using equations. There is the odd technical error, for example in the discussion of the Stern–Gerlach experiment, but these do not seriously impede the argument, and are compensated for by lucid discussions of the central points. I would put this book, along with Nick Herbert’s somewhat more technical *Quantum Reality*, at the top of my list of accounts of atomic-level quantum mechanics for the layman.

Now to the last quarter. Lindley is a forthright (and generally lucid) adherent of the school of thought that holds that the quantum measurement paradox is completely solved by a proper consideration of the phenomenon known nowadays as ‘decoherence’ — roughly speaking, the inevitable ‘entanglement’ of macroscopic systems with their environment and the resulting scrambling of phase relations. “Decoherence, though the idea has been around for a long time [it has indeed!], has only in the last decade or so become widely accepted as an essential ingredient in understanding the apparent definiteness and irreversibility of quantum mechanics.”

I think that Lindley here mistakes a sociological trend for a true intellectual development. Thirty years ago, John Bell remarked that “the typical physicist feels that [the questions related to the quantum measurement problem] have long been answered, and that he will fully understand just how if ever he can spare twenty minutes to think about it”. Since 1966, ‘decoherence’ has merely acquired an easily memorized name and been the subject of some elegant technical developments, some of them at the hands of people who were already household names in various

sub-fields of physics. Bell’s “typical physicist”, duly impressed by this star-studded cast, may now feel that even twenty minutes is superfluous.

If, however, we average over the community of serious students of the subject, including informed philosophers of science as well as the relevant minority of practising physicists, my impression is that discontent with the decoherence ‘solution’ has if anything increased over the past few years. This is certainly true in the United States, where conferences and workshops on the measurement problem are now being held with a frequency unthinkable fifteen years ago. (It is difficult to generate enthusiasm for meetings on problems generally regarded as solved.) Anyone who wants to know why should read Bell’s latest paper on the problem (*Physics World* 3(8), 33–40; 1990), in particular his trenchant insistence that no sleight of hand can replace an “and” by an “or”. Lindley refers to discontent on this point almost as an afterthought, only to confuse it with an Einstein-style hankering after determinism, a totally different issue; the central passage here utterly fails to address it, other than by simple fiat. He is, however, in distinguished company in missing what seems to me, and to a minority of other dissenters, a fundamental and crashingly obvious point. □

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## Chemicals unbottled

A. G. Cairns-Smith

**The Natural Selection of the Chemical Elements: The Environment and Life’s Chemistry.** By R. J. P. Williams and J. J. R. Fraústo da Silva. *Oxford University Press*: 1996. Pp. 646. £75, \$145.

OVERHEARD: “Chemical is it? Best leave it in the bottle. Nasty things chemicals.” But you can’t stopper them all. The bottle is ‘chemical’ too. So is the shelf it is on. So is everything else that makes up our extraordinary ‘ordinary world’.

Yes, but why are things around us made as they are, and of what they are? It is not always easy to say, but to provide a comprehensive guide to such answers was the heroic objective that Williams and Fraústo da Silva set themselves. The result is a *tour de force*, a major textbook of general chemistry, its mainly familiar topics reorganized under an obsessive theme. In this respect it is reminiscent of Pauling’s *The Nature of the Chemical Bond*, but the theme is different and the



setting wider.

The theme is selectivity. The approach is historical to begin with, arriving at the modern idea of the periodic table of elements. How do their atoms select and bond to each other? How do they form such specific higher-order structures as molecules or minerals, balancing order and disorder, being controlled by what gets made faster or by what is more stable? The second part of the book is a chemical history of Earth and life. Here there are questions of the selective factors at work in deciding, for example, which elements would predominate on the Earth. Part of this would have been in the accretion of components from the cloud of dust and gas from which the Solar System formed, with refractory materials favoured over volatile ones. Then the elements have been further corralled in atmosphere, ocean and crust. Here, as elsewhere in the book, phase separation is a crucial part of the story, a story of air, water, earth — and fire.

And then in life there would be new selective factors. What properties of the elements were relevant here for its origin, evolution or nanotechnology? Metal ions were always around, and no doubt always important for life; certainly they are today, in active centres of enzymes, as signals within and between cells, in bones and other biominerals.... The other aspects of life's molecular and cellular workings are also well described.

I have a gripe about the title, though. The book is all about selections at various levels, but it is a pity that these are discussed under the blanket term 'natural selection'. I would like to have ignored this as a minor gaffe — it could easily have been written out without altering the thrust of the book one jot — but there it is, blazoned in the title.

'Natural selection' is a technical term, short for a much longer explanation involving, for example, amplification through replication. The authors would never have used 'periodic table' to refer to a table that appears periodically, or 'free energy' to mean energy that one might get for nothing. So I don't think something should be described as natural selection just because it is natural and results in selection.

One consequence of this is that we may overlook the problem of the origin of life: the origin of a long-term process of evolution through, yes, *natural selection* — which is not just another example of the sort of thing we find here, there and everywhere. That would make the origin of life difficult in the boring sense that it would be hard to say in which of a zillion ways it might have happened. But actually the origin of life is difficult in an extremely interesting sense: it hardly seems possible that it could have hap-

pened at all. This is largely because natural selection depends on high-quality replication; finding molecular machinery that can do this under simple conditions is a major challenge for modern chemistry.

The final chapters consider man, his selection of materials for industrial purposes, and the global effects of his activities. These are not fundamentally different in scale from earlier disturbances by other forms of life. (Think of the first oxygen-producing photosynthe-

sis: now *there* was pollution.) But there is perhaps a difference for the future, if man develops a respect for the dead — for the 'dead' materials, as well as the living things in the world he inhabits.

My final impression? Chemistry is a wonderful subject. *The Natural Selection of the Chemical Elements* is a wonderful book. Pity about the title. □

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## The sins of science

John Emsley

**Blinded by the Sun.** A play by Stephen Poliakoff. Royal National Theatre (Cottesloe), London, until late December.

MONEY corrupts, power corrupts, fame corrupts. They have corrupted individuals in finance, politics and the media, and we are not really surprised because the corruptible are drawn to these areas, like wasps to jam. But what corrupts a scientist? Lack of money, lack of power, or lack of fame? Or could it be something else? Market forces even?

This ostensibly is the theme of Stephen Poliakoff's new play, *Blinded by the Sun*. It is set in the once-flourishing chemistry department of a northern university founded at the turn of the century. But times have changed. Science is in decline, and chemistry is no longer a fashionable subject to study; the department is under pressure, and its lacklustre staff no longer attract research funding.

The plot revolves round three characters: Albert ('Al'), Christopher and Elinor. Elinor (played by Frances de la Tour) is still acclaimed for her earlier work on vitamins, and is happy to do what she finds interesting, but hogs a disproportionate share of the department's resources. Christopher (Duncan Bell) is up and coming, but struggling to fund his cutting-edge research group. Al (Douglas Hodge) no longer does research, but is landed with the job of head of department.

What promises to transform this dusty department is the announcement by Christopher that he has found a catalyst for the photochemical decomposition of water into hydrogen gas by sunlight — and on an economic scale. Instead of patenting and publishing his results he decides to announce the discovery at a press conference: sound familiar?

Al senses that he has cheated, but which type of scientific cheating is it?

The sleight of hand of a downright fraud, or so wanting to demonstrate that the 'sun battery' works that he has cut a few corners?

We then jump forward ten years, to discover that, despite being in disgrace, Christopher is doing quite nicely. Elinor has not changed at all, apart from using nicotine patches instead of cigarettes. But Albert's time has come and he is in great demand, having written a scientific best-seller, *Beware of the Experts*, and has risen to the dizzy heights of giving those five-

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Elinor (Frances de la Tour).

minute talks on Radio 4 that come before the weather forecast. ("That's quite long enough for Albert's thoughts! Load of balls of course" is the emeritus professor's judgement.)

But in the new age of the market-place and the media, there is no place for small chemistry departments. Al has the seemingly unpleasant task of closing down the department to make way for media studies, and there is a particularly poignant scene in Elinor's laboratory when he finally tells her that she will have to retire. Their positions are now reversed, and when she says she must be allowed to continue her pure research, his retort is a cruel "the work is so pure, it's invisible". Even on bended knees Elinor fails to

move him.

*Blinded by the Sun* is a remarkable play, and Poliakoff has produced two roles that de la Tour and Hodge clearly relish and in which they shine. Even some of the minor parts, such as the emeritus professor (Graham Crowden), are gems.

The science itself sits lightly on the plot, and might irritate the odd academic purist, but this hardly matters. It matters a little more that the issue of scientific fraud is not fully explored, and I was expecting that at some stage Christopher would be confronted and asked to explain why he cheated. Despite this omission, Poliakoff is to be congratulated on portraying chemists as real people, ill-equipped to wrestle with the complex pressures of a discipline in decline.

Fifty years ago, chemistry was in its ascendancy, and then it was lovingly portrayed by Alec Guinness in the Ealing comedy *The Man in a White Suit*. Since those heady days it has been almost ignored by writers and dramatists. Per-

haps if we had reached out to communicate better, they might have continued to be our allies, and we would not be in the unenviable position in which we now find ourselves.

Britain has more than 75 chemistry departments, all in theory able to do research and award PhDs. Many are under the pressures explored in *Blinded By the Sun*. Many are fated to close within the next ten years, unable to shake off their Elinors, unable to generate new funding for their Christophers, and unable to accommodate the success of their Als.

These are the real issues around which the play is built, not the apparent one of the unthinkable sin of scientific fraud. Perhaps Poliakoff set too many hares running, and this one got away, but that is a minor flaw in what is otherwise a superb play. □

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## Choosing genes

Alan F. Wright and A. Christopher Boyd

**The Gift.** A play by Nicola Baldwin. Currently touring in the UK until 2 November. Details from Y Touring, 8-10 Lennox Road, London N4 3NW, UK.

DISCOVERING ourselves is part of growing up, and for most it is not all good news. For the Kay family, the process is sudden and painful. Annie, a talented, athletic teenager, discovers she has the incurable, hereditary neurodegenerative disease Friedreich's ataxia. Her younger brother, Ryan, a "nerdy scruffbag with zits", wants to undergo the genetic test but is too young to be screened. The devastated Kays, as if to vent their fury at what for Annie is "a death sentence, no, worse, a life sentence", rally to Ryan's cause — the right to know. They win their case, and Ryan is found to be an unaffected carrier of Friedreich's ataxia.

The plot thickens when the action jumps ahead 15 years, and his wife also turns out to be a carrier, giving them a one-in-four risk of an affected child. Genetic profiling and *in vitro* fertilization are available, but she wants a child whose genetic constitution is determined by chance alone. Ryan, now a geneticist, deceives his wife (who eventually agreed to select out only Friedreich's ataxia) by choosing a particular disease-free embryo to fulfil not just his own but also his now-dead sister's dreams: a male predisposed to athleticism.

The action jumps a further 15 years to Ryan's son's horrified discovery of his genetic destiny. The ironic consequence

of the father's decision emerges — the son so hates the knowledge that his hard-won tennis achievements were predestined that he walks out on both his training and his father.

*The Gift* develops these themes to highlight the issues raised by our burgeoning knowledge of human genetics. Insights

message is "you decide".

Commissioned by the Wellcome Centre for Medical Science, *The Gift* is aimed primarily at teenagers, but the issues raised are of such general concern that it deserves a wider audience. The play succeeds admirably both as pure theatre and in its aim to inform the public. No longer just an esoteric science, genetics concerns us all: it is about life and death, the meaning of and response to disability, and the new moral dilemmas created by increased knowledge.

At the end, the audience is drawn into debate and asked to vote on the extent to which genetic make-up should be a matter of choice. The all-too-short opportunity to challenge the characters, and an invited geneticist, is enthusiastically seized. The idea of selection against embryos carrying a fatal disease was finally accepted by Ryan's wife, but she would have had grave difficulties with less clear-cut choices. This was also the verdict of most of the audience.

The deliberate selection of human genes for complex traits such as athleticism is a distant prospect, but not wholly implausible; we know already that such selection would have dangerous and unpredictable consequences. As George Bernard Shaw allegedly said to Isadora Duncan, "but what if it has *my* looks and *your* brains?" In contrast, we already have formidable knowledge of genetic disease susceptibility. Some geneticists argue against the wide availability of screening for incurable disorders, but the public may demand "the right to know". As Annie says, "sometimes knowledge is a cure in itself", but it can be an agonizing one.

Widely lauded before and during its run at the Edinburgh Festival, *The Gift* played to packed halls. The audience was captivated and responded positively because the complex issues were so deftly handled. For some, the idea of a father choosing a genetic "gift" for his son is repellent; for others, it sends all the wrong messages.

Designer genes for successful living devalue the roles of social inheritance and personal freedom, and may even alienate the beneficiary: the son rejects the "gift" and leaves to forge his own destiny. The final irony is Ryan's admission that he could not contemplate the idea of selecting against Annie: "I wouldn't have changed a thing — except the rogue gene". *The Gift* is as much about relationships, disability, feelings and possibilities as it is about the genes that limit us. It serves as an inspiring model for future productions with similar aims, and is an education for us all. □

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IMAGE  
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Annie Kay (Germaine Elliott).

and quandaries are presented at every turn, yet the play manages to be funny, entertaining and deeply moving. The non-judgemental line taken is especially commendable: while the characters represent a wide spectrum of opinion, the overall

Robert Workman

# Supercritical fluids as solvents for chemical and materials processing

Charles A. Eckert, Barbara L. Knutson & Pablo G. Debenedetti

**Fluids near their critical point have dissolving power comparable to that of liquids, are much more compressible than dilute gases, and have transport properties intermediate between gas- and liquid-like. This unusual combination of physical properties can be advantageously exploited in environmentally benign separation and reaction processes, as well as for new kinds of materials processing.**

ALTHOUGH the ability of supercritical fluids (SCFs) to dissolve non-volatile solids has been known for more than a century<sup>1</sup>, interest in their commercial exploitation has occurred only in the past twenty years. This interest was first motivated during the early 1970s by concerns about energy costs and by the idea (since proved incorrect) that separations using supercritical solvents might be attractive energy-saving alternatives to distillation and liquid extraction. The capability of some supercritical fluids for replacing toxic industrial solvents<sup>2,3</sup>, the ability to make new materials at mild conditions<sup>4,5</sup> and the possibility of tuning solvent characteristics for highly specific reactions or separations<sup>6</sup> are the main considerations that underlie current industrial and scientific interest in supercritical fluids.

Large-scale supercritical extraction is well established, examples being the decaffeination of coffee with supercritical CO<sub>2</sub> (ref. 7) and the ROSE process for separating the components of the heavy fraction of petroleum using supercritical pentane<sup>8</sup>. A plethora of smaller-scale applications exist, such as the extraction of drugs from plant materials<sup>9</sup>, and the processing of heat-sensitive pharmaceutical compounds, which benefits from both the mild operating temperatures (usually in CO<sub>2</sub>) and the recovery of a dry product requiring no further solvent separation. McHugh and Krukoni<sup>10</sup> give a comprehensive review.

A fluid is supercritical when its temperature and pressure are higher than their critical-point values ( $T_c$ ,  $P_c$ ). Most interesting applications of SCFs occur in the range  $1 < T/T_c < 1.1$  and  $1 < P/P_c < 2$  (ref. 11). At these conditions, the fluid exists as a single phase having some of the advantageous properties of both a liquid and a gas: it has sufficient density to give appreciable dissolving power; but the diffusivity of solutes in SCFs is higher than in liquids, and the viscosity of SCFs is lower, facilitating mass transport. Finally, because of the high compressibility of fluids near the critical point, their density and dissolving power can be tuned sensitively through small changes in pressure.

Most applications of SCFs involve dilute mixtures composed of a small-molecule volatile supercritical solvent and one or more solutes of low volatility differing appreciably from the solvent in mass, size, interaction strength, polarity and shape. These mixtures have interesting and complex properties because of the different behaviours of the components and the solvent's proximity to its critical point<sup>12</sup>. For example, the solubility of solutes is orders of magnitude larger than in ideal gases; enhancements of  $10^4$ – $10^8$  relative to ideal-gas solubilities are not uncommon. Solute solubility depends exponentially on density<sup>13,14</sup> (Fig. 1), and small pressure changes in the highly compressible near-critical region can cause very large solubility variations, allowing the selective dissolution or precipitation of solutes.

From a theoretical perspective, nonidealities arising from the disparity between solvent and solutes, coupled with the effect of density fluctuations due to proximity to the critical point, limit the

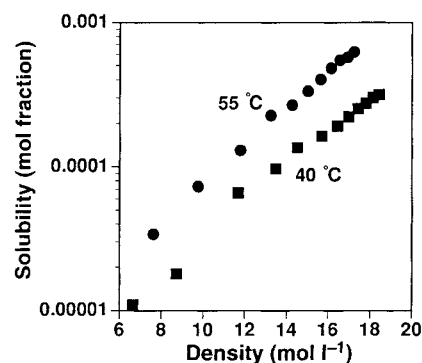


FIG. 1 Solubility of salicylic acid in supercritical CO<sub>2</sub>. Data from ref. 14.

applicability of traditional approaches for modelling phase equilibria. So there is still much to be learned from theoretical and computational studies about solvation in near-critical solvents.

Although only a limited number of SCFs are in common use, they are often tailored for specific applications by adding cosolvents. Bulk fluid characteristics such as solvent power towards a particular compound can be changed appreciably by addition of a small amount of a volatile cosolvent, usually a polar or protic species such as a small alcohol or acetone<sup>15–17</sup>. While this affects the solution's critical properties slightly, it may greatly enhance the solvent's loading capacity or selectivity. To increase the separation efficiency, cosolvents that are capable of specific interactions with the solutes of interest are often chosen (Fig. 2)<sup>18</sup>. Similarly large effects on the selectivity occur when multiple solutes are present<sup>19</sup>.

In spite of their many attractive characteristics, SCFs are not a panacea. If a separation can be carried out by traditional methods, such as liquid extraction or adsorption, it is generally less expensive than by SCF processing. Moreover, as SCFs are typically less dense than liquids by factors of 1.5–3, their solvent power, although appreciable, is smaller than that of the corresponding liquid. Furthermore, the cost associated with the compression and containment of the fluid may limit the economic feasibility of the SCF extraction process. Thus the most promising applications of SCFs are those in which their unusual properties are exploited towards making products with characteristics and specifications that cannot be obtained by other means.

## Physical properties

What makes SCFs and their mixtures scientifically interesting is the existence of widely different relevant length scales. Long-



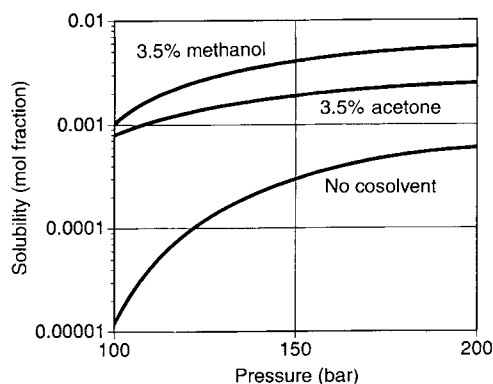


FIG. 2 Cosolvent effects for salicylic acid in CO<sub>2</sub> at 55 °C. Data from ref. 18.

ranged correlations caused by proximity to criticality underlie the large compressibilities characteristic of these systems and influence the rate of change of solubilities with respect to temperature and pressure. On the other hand, solvation effects due to molecular disparity are short-ranged and unrelated to criticality, and these control the absolute solubility.

**Pure fluids.** The isothermal compressibility of any pure fluid,  $K_T = (1/\rho)(\partial\rho/\partial P)_T$ , where  $\rho$  is the density, is infinite at the critical point and is very large under conditions commonly encountered in practical applications of SCFs. As  $K_T$  is proportional to the mean squared density fluctuations, the latter diverge at the critical point, and are large under commonly encountered conditions. In addition,  $K_T$  is proportional to  $\int r^2[g(r) - 1]dr$ , where  $g(r)$  (the pair correlation function) is the ratio of local to bulk density at a distance  $r$  away from a molecule fixed at the origin. The divergence of  $K_T$  at the critical point is therefore a consequence of  $g(r)$ , a finite quantity, becoming long-ranged (Fig. 3)<sup>20</sup>.

The quantity  $\xi$  measures the range of density fluctuations, and is called the correlation length. The decay of the pair correlation function near (but not at) the critical point is described by:

$$g(r) \propto \frac{\exp(-r/\xi)}{r} \quad (1)$$

The correlation length of any fluid diverges at the critical point, and it far exceeds molecular dimensions in its vicinity. Using carbon dioxide at its critical density as an example<sup>20,21</sup>,  $\xi = 55$  Å 1 K above  $T_c$ , and  $\xi = 13$  Å 10 K above  $T_c$ . The average intermolecular distance at the critical density, on the other hand, is 5.4 Å. Thus the short-ranged details of intermolecular forces are not related to the fluctuation-dominated long-range behaviour of the correlation function, the growth in the compressibility, or the increase of the correlation length near the critical point.

The viscosity  $\eta$  of SCFs exhibits a weak divergence that can be observed only in the immediate vicinity of the critical point<sup>22</sup>. Under conditions encountered in applications, the viscosity of SCFs decreases with temperature at constant pressure, whereas that of low-density gases increases as  $T^{1/2}$  and is insensitive to pressure. Compared to the gas at the same temperature, the viscosity of a supercritical fluid is typically less than one order of magnitude higher, but its density is at least two orders of magnitude higher. Consequently, the ratio of these two quantities, the kinematic viscosity  $\nu = \eta/\rho$ , is very low in the supercritical region. This is important for mass-transfer applications, because natural convection effects are inversely proportional to the square of the kinematic viscosity<sup>23</sup>. Diffusion coefficients at infinite dilution of solutes in supercritical fluids are typically one or more orders of magnitude greater than in liquids at the same temperature<sup>24</sup>. Table 1 gives characteristic magnitudes of some thermophysical properties of gases, liquids and supercritical fluids.

**Dilute mixtures.** Dilute binary mixtures are important in many

applications of supercritical fluids. A useful classification scheme recognizes three categories<sup>25</sup>: attractive, repulsive and weakly attractive. All known applications of supercritical fluids involve attractive mixtures, where the solute is generally much less volatile and larger than the solvent. At infinite dilution, the solute's partial molar volume  $\bar{v}_2^\infty$  and enthalpy  $\bar{h}_2^\infty$  (that is, the rate of change of the mixture's volume and enthalpy with respect to solute addition at constant temperature and pressure) diverge at the solvent's critical point, to  $-\infty$  in attractive mixtures, and to  $+\infty$  in repulsive ones. Dilute attractive mixtures are thus characterized by large and negative solute partial molar volumes and enthalpies near the solvent's critical point and over an appreciable range of supercritical pressures. The divergence  $\bar{v}_2^\infty$  and  $\bar{h}_2^\infty$  is due exclusively to the growth of the correlation length near the critical point (Fig. 4). This follows from the fact that both quantities are proportional to the solvent's compressibility<sup>20,25</sup>.

Typical systems of practical interest often consist of a solute (solid at ambient conditions) in a dilute solution in an SCF. In these systems, the mixture's lower critical end point (LCEP, where the difference between solvent-rich liquid and vapour phases disappears) is close to the solvent's critical point. Thermodynamic arguments show that above the LCEP the solubility increases with pressure at constant temperature, and decreases with temperature at constant pressure<sup>20</sup>.

In most supercritical mixtures of practical interest, the solute(s) and the solvent differ appreciably in size, shape, interaction strength and polarity. This asymmetry gives rise to interesting solvation effects not ordinarily found in liquid mixtures<sup>26-29</sup>. The characteristic energies for solute-solute and solute-solvent interactions are much stronger than for solvent-solvent interactions. Hence the local environment around the solute can differ appreciably in density and composition from the bulk. This local-bulk anisotropy is unrelated to proximity to critical points, and makes it possible to tailor the composition and density of local environments for specific reaction or separation applications through the use of cosolvents<sup>29</sup>.

## Experimental and computational studies

Many techniques are available for studying supercritical mixtures. Detailed knowledge about solute-solute, solute-cosolvent, cosolvent-solvent and solute-solvent molecular interactions is essential for developing predictive models of supercritical behaviour.

**Thermodynamic measurements.** These have been made on the solubility of solids (see, for example, ref. 30), and on partial molar properties of dilute solutes in near-critical solvents (for example, refs 31 and 32). In the former case, interest lies in the remarkable solubility enhancements relative to the dilute gas limit, which can

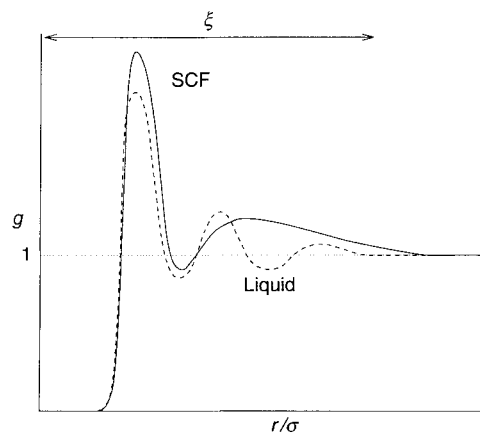


FIG. 3 Schematic representation of the pair correlation function in liquids and in supercritical fluids.  $\xi$  is the correlation length,  $g$  is the ratio of local to bulk density a distance  $r$  away from a molecule fixed at the origin, and  $\sigma$  is the molecular diameter. (Adapted from ref. 20.)

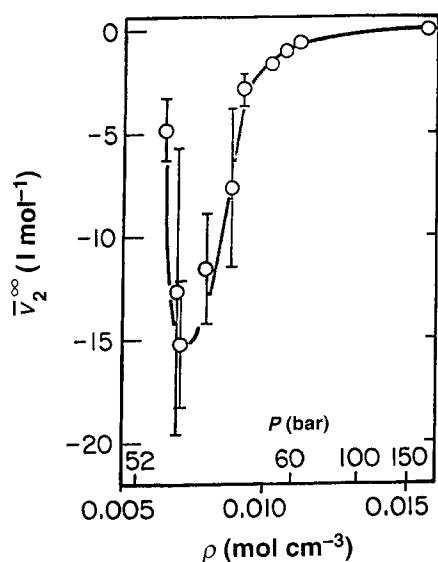


FIG. 4 Infinite dilution partial molar volume of naphthalene in supercritical ethylene ( $T_c = 282.4$  K,  $\rho_c = 0.0077$  mol cm $^{-3}$ ) at 285 K (ref. 31).

be as large as  $10^{12}$  (ref. 11). In the latter case, improved experimental techniques have made it possible to measure the effects of critical anomalies on bulk thermodynamic properties of supercritical mixtures, in agreement with previous theoretical calculations<sup>33</sup>.

When extracting solutes from a mixed-solid bed, a mutual solubility increase relative to the pure solid solubilities was observed by Kurnik and Reid<sup>34</sup>. Specifically, they measured solubility enhancements of 280% and 107% for the mixed solute extraction of benzoic acid and naphthalene, respectively, in supercritical CO<sub>2</sub>. Such a mutual solubility enhancement is expected<sup>35</sup> for dilute attractive mixtures, although at least one counter-example has been reported<sup>36</sup>.

Similarly, the addition of a small amount of a volatile cosolvent (usually less than 5 mol%) to a supercritical fluid solution may dramatically alter solute solubility. Theoretical arguments for highly dilute solute–cosolvent systems in a near-critical solvent indicate that the enhanced solubility is due to solute–solvent and cosolvent–solvent interactions<sup>35</sup>. The systems that have been studied experimentally, however, are not infinitely dilute (they typically contain 5 mol% cosolvent), nor is the solvent in the immediate vicinity of its critical point. In such systems, solute–cosolvent specific interactions, such as hydrogen bonding, charge-transfer complex formation, and dipole–dipole coupling appear

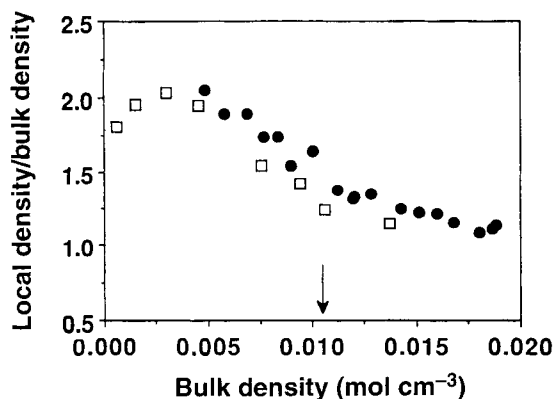
to be important<sup>29</sup>. As shown in Fig. 2, polar protic substances have a substantial cosolvent effect (as great as a factor of approximately 100 increase in solubility) on salicylic acid<sup>18</sup>. Only some of the solubility enhancement can be attributed to the bulk density increase with addition of cosolvent; extreme cosolvent effects are generally associated with specific solute–cosolvent interactions.

**Spectroscopic measurements.** Spectroscopic techniques provide information on solvation (short-ranged solute–solvent, solute–cosolvent and solute–solute interactions) and solvent structure. With the correct match of probe molecule and spectroscopic method (for example, ultraviolet absorbance, fluorescence emission, infrared, electron spin resonance and nuclear magnetic resonance spectroscopies), the environment near the probe can be examined through energy shifts, changes in intensity or spectral features signifying the formation of complexes. Investigations in supercritical fluids have relied on well characterized probes of liquid systems to obtain qualitative information on the local environment around solute molecules (the microstructure of solvation).

Pyrene is a good spectroscopic probe molecule for quantifying the enhancements in solvent density around the solute relative to bulk conditions, because features of the fluorescence from pyrene are very sensitive to short-ranged interactions with the solvent. The solvent-sensitive intensity ratio of the first and third fluorescence peaks is the basis of the pyrene (Py) scale of solvent strength in liquids<sup>37</sup>, and this scale has been used to quantify enhancements in SCF solvent density<sup>27,38</sup>. Appreciable enhancements of up to 2.5 times the bulk density were seen for supercritical C<sub>2</sub>H<sub>4</sub>, CHF<sub>3</sub> and CO<sub>2</sub> around pyrene<sup>27</sup>. Figure 5 compares local solvent densities around a dilute pyrene molecule in CO<sub>2</sub> obtained from fluorescence spectroscopy and from molecular-dynamics simulations of a Lennard–Jones mixture<sup>38</sup>. The ratio of local to bulk density is approximately 1.3 in near-critical carbon dioxide, and reaches a maximum of about 2 below the critical density (at  $\rho_t = \rho/\rho_c \approx 0.5$ )<sup>38,39</sup>.

For investigating solvation in cosolvent-modified supercritical fluid mixtures, solvatochromic probe molecules (which have an ultraviolet–visible absorption band whose position is sensitive to the solvent environment) have been used. Local compositions have been inferred from solvatochromic shifts of phenol blue in supercritical CO<sub>2</sub> with the cosolvents methanol, ethanol, acetone and *n*-octane (ref. 40). The composition in the immediate vicinity of the probe was quantified by assuming that the solvent and cosolvent contribute linearly to the shift based on their local mole fraction<sup>40</sup>. For the cosolvents investigated, the inferred local composition (in mole fraction units) was greater by a factor of approximately 7 than the bulk composition for all 1 mol% (bulk) cosolvent mixtures at 80 bar and 35 °C. A similar enrichment of cosolvent was observed for the system 2-nitroanisole/2-propanol/CO<sub>2</sub> (ref. 41). These results suggest that significant differences exist between local and bulk compositions in cosolvent-modified

FIG. 5 Comparison between local CO<sub>2</sub> (solvent) density around dilute pyrene quantified by fluorescence spectroscopy (●) and the corresponding molecular-dynamics results (□) at a solvent reduced temperature of 1.02 (ref. 38). The simulation numbers are based on a sphere of radius 7.4 Å around the solute, corresponding to a solvation shell of thickness equal to one solvent diameter.



mixtures. This phenomenon is not, however, unique to supercritical solutions<sup>42</sup>.

Hydrogen-bonding interactions in SCFs are particularly well suited for direct quantification by Fourier transform infrared (FTIR) spectroscopy<sup>43,44</sup>. The degree of solute–solute hydrogen bonding in supercritical CO<sub>2</sub> and C<sub>2</sub>H<sub>6</sub> systems containing up to 0.07 mole fraction methyl alcohol-*d* was investigated over a broad range of subcritical and supercritical conditions<sup>43</sup>. Spectral evidence suggests the formation of a weak alcohol–CO<sub>2</sub> complex, which shifts the alcohol monomer/oligomer equilibrium towards monomer formation.

In spite of the appreciable enhancements in the local solvent density and cosolvent composition around non-volatile solutes, especially at subcritical density and supercritical temperatures, spectroscopic investigations of complex formation suggest that the solute microstructure lacks the temporal persistence and structural integrity of liquid-phase hydrogen bonds. For example, in the system 7-azaindole solute/methanol cosolvent/CO<sub>2</sub>, although fluorescence of the excited-state azaindole/alcohol complex suggests extensive interaction between these species<sup>45</sup>, the 7-azaindole tautomer is not observed, indicating the absence of a hydrogen-bonded network needed to stabilize the complex in structured liquids. This lack of structure is consistent with spectroscopic investigations of the solvent-sensitive proton transfer in 2-naphthol and its cyano-derivatives<sup>45</sup>.

**Computational studies.** Unlike spectroscopic techniques, computational methods provide detailed information on the number of molecules in a precisely defined region around a given molecule. Hence they are very useful in the study of short-ranged interactions and solvation phenomena in supercritical solvents. Two approaches are commonly used: molecular-dynamics simulation<sup>46</sup> and integral-equation calculations<sup>47–53</sup>. Molecular dynamics gives essentially exact answers for a given intermolecular potential and it also provides time-dependent information. The study of dilute mixtures by molecular dynamics is quite challenging because it requires large system sizes (10<sup>4</sup> to 10<sup>5</sup> molecules) in order to gather good statistics for solute–solvent interactions at the low solute mole fractions characteristic of supercritical applications. In contrast, integral equations are ideally suited to study dilute mixtures. Both techniques fail in the immediate vicinity of the critical point. Molecular dynamics fails because the increase of the correlation length requires the use of very large systems, because the exact location of the critical point of the simulated system is uncertain, and because of the long equilibration times required at near-critical conditions. Integral equations predict mean-field analytical behaviour near the critical point, in contrast with experimental observations for real fluids<sup>21</sup>.

To date, most simulation and integral equation calculations have used simplified, spherically symmetric potentials (for example, Lennard–Jones mixtures) to study supercritical systems. Results therefore represent ideals of molecular interactions which do not capture all of the structural and dynamic effects associated with the complex molecules of typical SCF solutions. In spite of these approximations, this work has been invaluable in characterizing solvation in highly asymmetric systems, as illustrated by the comparison in Fig. 5. More recently, molecular-dynamics simulations using realistic potentials have been used to study solvation and reactions in supercritical water<sup>55–56</sup>.

**Kinetic experiments.** Supercritical fluids are attractive media for reactions because they allow the sensitive control of phase behaviour<sup>57</sup> (dissolution of reactants or precipitation of products) with moderate changes in temperature and pressure, and because they offer the opportunity to fine-tune reaction rate and selectivity with very small changes in operating conditions.

The pressure-dependence of a reaction rate is proportional to the activation volume,  $\Delta v^\ddagger$ :

$$\left(\frac{\partial \ln k_r}{\partial P}\right)_T = -\frac{\Delta v^\ddagger}{RT} \quad (2)$$

which is the difference in partial molar volumes of the transition

TABLE 1 Characteristic magnitudes of thermophysical properties

|        | $\rho$ (kg m <sup>-3</sup> ) | $\eta$ (cP)      | $D^*$ (cm <sup>2</sup> s <sup>-1</sup> ) |
|--------|------------------------------|------------------|------------------------------------------|
| Gas†   | 1                            | 10 <sup>-2</sup> | 10 <sup>-1</sup>                         |
| SCF    | 300–800                      | 0.03–0.1         | 10 <sup>-4</sup>                         |
| Liquid | 10 <sup>3</sup>              | 1                | 10 <sup>-5</sup>                         |

Symbols used:  $\rho$ , density;  $\eta$ , viscosity;  $D$ , diffusion coefficient. SCF, supercritical fluid.

\* Diffusion coefficient of small-molecule solute in given fluid.

† At ambient conditions.

state and the reactants. Thus, for a bimolecular reaction,

$$\Delta v^\ddagger = \bar{v}_M - \bar{v}_A - \bar{v}_B \quad (3)$$

where M, A and B refer to the reaction transition state and the two reactants, respectively. The rate constant,  $k_r$ , is expressed in mole fraction units. In liquids,  $\Delta v^\ddagger$  is typically  $\pm 0$ –40 cm<sup>3</sup> mol<sup>-1</sup>. In attractive supercritical mixtures, values of  $\Delta v^\ddagger$  are of the order of litres per mol owing to the large negative partial molar volumes of the reactants and transition states near the solvent's critical point<sup>58</sup>. The pressure dependence of reaction rates is a consequence of the solvent's large compressibility; the dependence is smooth if plotted against density.

Reaction selectivities in supercritical fluids are also pressure-sensitive<sup>59</sup>. Local density and composition effects influence reaction rates and selectivities in supercritical fluids by changing the relative stabilization of reactants and transition states. In certain bimolecular reactions, apparent rate constants may reflect the local composition around a dilute reactant, which is enriched in reactive cosolvent with respect to bulk conditions<sup>60,61</sup>. The bimolecular reaction rate constant of dilute phthalic anhydride with a reactive cosolvent (methanol) in supercritical CO<sub>2</sub> shows a 25-fold decrease with increasing pressure (97.5 to 166.5 bar) when calculated on the basis of the bulk composition of methanol<sup>60</sup>, whereas transition-state theory predicts only a twofold decrease for that increase in pressure. This discrepancy suggests a considerable enhancement of cosolvent in the vicinity of the dilute reactant at the lowest pressures investigated. Knowledge of this local reactant mole fraction is necessary to obtain true rate constants in these solutions. Spectroscopic results for methanol around phenol blue solute in supercritical CO<sub>2</sub> show that the local composition enhancement is of the same magnitude that would be required to create the observed discrepancy<sup>60</sup>.

A variety of kinetic investigations have examined the influence of local effects on diffusion-controlled reactions. Evidence exists in support of both the presence<sup>62,63</sup> and absence<sup>63–65</sup> of solute–solvent and solute–solvent cage effects in diffusion-controlled reactions. Randolph *et al.*<sup>66</sup> clarified these apparently conflicting observations in a molecular-dynamics investigation of solute–solvent collision and reaction rates. They showed that at low bulk density the dependence of the solute–solvent bimolecular reaction rate constant on collision diameter is identical to that of the radial distribution function, and therefore that local composition effects have a pronounced influence on the rate constant. At high density, solute–solvent encounters become diffusion-limited and local composition effects disappear. The transition from collision-limited to diffusion-limited behaviour is system-specific.

## Applications

Innovative uses of supercritical fluids as reaction solvents have recently been reported. Beckman, Russell and co-workers used pressure to control enzyme activity in a variety of supercritical solvents<sup>67</sup>. These results demonstrate the feasibility of producing chiral compounds in SCFs, and of using the tunable nature of these solvents to maximize selectivity to the desired stereoisomer. Beckman and Russell were also able to use pressure changes to adjust polymer molecular weight in biocatalytic synthesis in supercritical solvents<sup>68</sup>. In this example, the tunable solvent strength of



the fluid dictates the product molecular weight and distribution; as the reaction proceeds, a polymer molecular weight is reached at which the polymer precipitates. DeSimone and co-workers have used novel approaches to the problem of finding environmentally benign alternatives to commonly used organic solvents in polymerization reactions<sup>2,3</sup>. They have synthesized high-molecular-weight homo- and copolymers of fluorinated monomers in supercritical CO<sub>2</sub> by free-radical methods<sup>2</sup>. They have also designed amphipathic molecules with 'CO<sub>2</sub>-philic' and lipophilic moieties and used them to stabilize the dispersion polymerization of lipophilic methyl methacrylate to high-molecular-weight product in supercritical CO<sub>2</sub> (ref. 3).

Another intriguing use of supercritical fluids as reaction media involves phase-transfer catalysis (PTC). To achieve reactions between species of very different polarities, such as nonpolar molecules with ionic species, requires solvents that may be expensive, undesirable environmentally and difficult to remove. PTC is an important and effective method for conducting such heterogeneous reactions using a catalyst (quaternary ammonium salts and cyclic ethers) to transport ionic reactants into a nonpolar phase. For example, the bromination of benzyl chloride by KBr appears to be a simple reaction, but the salt is insoluble in the organic phase and the reaction at the interface between two phases is extremely slow. Acetone as a cosolvent permitted the dissolution in supercritical CO<sub>2</sub> of sufficient (C<sub>7</sub>H<sub>15</sub>)<sub>4</sub>N<sup>+</sup>Br<sup>-</sup> to catalyse this reaction<sup>6</sup>.

In general, the use of SCF solvents offers several advantages for heterogeneous reaction processes:

- (1) Environmentally benign solvents such as cheap, non-toxic, non-flammable CO<sub>2</sub>, which can be used pure or with small amounts of modifiers.
- (2) Ease of solvent removal.
- (3) Reduction of mass transfer limitations (see Table 1)
- (4) Milder reaction conditions. Use of solvents with moderate critical temperatures, such as CO<sub>2</sub> (31 °C), combined with extreme sensitivity of reaction rates to pressure allows fast rates to be obtained at mild conditions.
- (5) Higher yield and selectivity. Temperature, pressure and cosolvents can be used to tailor rates and selectivities in chemical reactions.

Materials processing is also a promising area of application of supercritical fluid technology. Complementary techniques exist for the formation of particulate phases starting from supercritical fluids: the rapid expansion of supercritical solutions (RESS) and the supercritical anti-solvent (SAS) technique. RESS involves the rapid depressurization of a supercritical fluid phase in which the solute(s) of interest is(are) dissolved. Small particles are obtained owing to the large supersaturations associated with the rapid loss of density and solvent strength in the compressible fluid phase. In SAS, the solute(s) is(are) dissolved in a liquid organic phase. Precipitation is achieved by bringing this solution into contact with a supercritical fluid having a low affinity for the solutes and a large mutual solubility with the organic phase. SAS overcomes the primary limitation of RESS, which is the low solubility of many polymeric<sup>5,69</sup> and biological<sup>70-72</sup> solutes in common supercritical solvents. Both processes produce dry particles in a single processing step and lead to direct solvent recovery. Another useful variation is precipitation with a compressed fluid antisolvent (PCA)<sup>73</sup>, in which the antisolvent can be injected at subcritical conditions. PCA has been used to produce a wide variety of polymer morphologies<sup>73</sup>.

RESS and SAS are well suited to pharmaceutical applications because of the mild operating conditions and the purity of the products. RESS has been used to produce pure and composite particles of a variety of materials, including ultrafine pharmaceutical powders (see ref. 74 for a review). The feasibility of coprecipitating drugs and low-molecular-weight bioerodible polymers to form drug-loaded microparticles for controlled release applications has also been investigated<sup>4</sup>. Pharmaceutical applications of SAS include the processing of proteins<sup>71,72</sup> and bioerodible poly-

mers<sup>75</sup>. In the former case, protein powders suitable for controlled release and delivery in aerosol form to the lungs have been produced, with virtually complete retention of biological activity on redissolution<sup>70-72</sup>.

In addition to the environmentally driven replacement of organic solvents, supercritical fluids have many direct environmental applications in soil, water and waste remediation. Soils and sludges contaminated with organics have been treated with supercritical CO<sub>2</sub> (ref. 76). This solvent has also been used to regenerate granular activated carbon which is used to purify waste waters, ground waters and leachates (see, for example, ref. 77). Another significant environmental application is the wet oxidation of organic contaminants in supercritical water, an alternative to incineration, which has been studied extensively (see ref. 78 for a review). Both oxygen and organics are completely soluble in supercritical water, which has a low dielectric constant. Organic contaminants are oxidized completely (>99.99%) in a few seconds to form small product molecules such as H<sub>2</sub>O and CO<sub>2</sub>.

SCFs can be feasible alternatives to conventional solvent systems when they are associated with the production of high-value-added products (for example, in the pharmaceutical industry), in applications where environmental concerns are of primary importance (organic-solvent-free polymer synthesis), or when the possibility of coupling processing steps exist (for example, reaction and product separation). The use of supercritical fluids to process or synthesize biological and polymeric solutes at mild conditions and to carry out reactions in environmentally responsible ways is especially promising.

## Prospects

What then might be the future be for this technology? Most applications will involve relatively low-volume, high-value-added products where the cost of SCF processing is more than offset by processing advantages or product quality or novelty. These applications will include novel extractions—many are already being done in the food, flavour and pharmaceutical industries, but remain proprietary. There will be increased use of SCFs for chemical reactions, to improve mass transfer, selectivity and conversion, or perhaps to yield selected polymers or chiral products. Catalysis in SCFs will become more important, not only with phase-transfer catalysis, but also with compounds such as transition-metal complexes, which are CO<sub>2</sub>-soluble<sup>79</sup>. Furthermore, progress in the development of CO<sub>2</sub> surfactants has led to rapid advances in the ability to do aqueous chemistry in microemulsions in SCF CO<sub>2</sub>, opening up new possibilities for bio-reactions<sup>80,81</sup>. In addition, the particle-forming techniques of RESS and SAS are being applied not only to encapsulation for drug delivery, but also to other areas ranging from ceramic precursors to propellants. SCFs have broad applications in tailoring both the chemistry and physics of polymers—by dissolving, swelling, or facilitating transport<sup>82</sup>. Surely even more opportunities will come to the fore as our fundamental understanding of SCF chemistry improves, and as process experience improves the reliability of scale-up and design. □

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1. Hannay, J. B. & Hogarth, J. *Proc. R. Soc. Lond. A* **29**, 324–326 (1879).
2. DeSimone, J. M., Guan, Z. & Elsbernd, C. S. *Science* **257**, 945–947 (1992).
3. DeSimone, J. M. et al. *Science* **265**, 356–359 (1994).
4. Tom, J. W., Lim, G.-B., DeBenedetti, P. G. & Prud'homme, R. K. in *Supercritical Fluid Engineering Science: Fundamentals and Applications* (eds Kiran, E. & Brennecke, J. F.) 238–257 (ACS Symp. Ser. 514, American Chemical Soc., Washington DC, 1993).
5. Dixon, D. J. & Johnston, K. P. *J. Appl. Polym. Sci.* **50**, 1929–1942 (1993).
6. Dillow, A. K. et al. *Ind. Eng. Chem. Res.* **35**, 1801–1806 (1996).
7. Zosel, K. *Angew. Chem. Int. Edn Engl.* **17**, 702–709 (1978).
8. Gearhart, J. A. & Garwin, L. *Hydrocarbon Process.* **55**, 125–129 (1976).

9. Schaeffer, S. T., Zalkow, L. H. & Teja, A. S. *Biotechnol. Bioeng.* **34**, 1357–1365 (1989).
10. McHugh, M. & Krukonis, V. *Supercritical Fluid Extraction* (Butterworth-Heinemann, Boston, 1994).
11. Brennecke, J. F. & Eckert, C. A. *Am. Inst. Chem. Eng. J.* **35**, 1409–1427 (1989).
12. Schneider, G. M. in *Supercritical Fluids, Fundamentals for Application* (eds Kiran, E. & Levett Sengers, J. M. H.) 91–116, 739–760 (NATO ASI Ser. E, 273, Kluwer, Dordrecht, 1994).
13. Kumar, S. K. & Johnston, K. P. *J. Supercrit. Fluids* **1**, 15–22 (1988).
14. Gurdial, G. S. & Foster, N. R. *Ind. Eng. Chem. Res.* **30**, 575–580 (1991).
15. Brunner, G. & Peter, S. *Ger. Chem. Eng.* **5**, 181–195 (1982).
16. Schmitt, W. J. & Reid, R. C. *Fluid Phase Equil.* **32**, 77–99 (1986).
17. Brunner, E. J. *Chem. Thermodyn.* **17**, 671–679 (1985).
18. Gurdial, G. S., Macnaughton, S. J., Tomasko, D. L. & Foster, N. R. *Ind. Eng. Chem. Res.* **32**, 1488–1497 (1993).
19. Van Alsten, J. G. & Eckert, C. A. *J. Chem. Eng. Data* **38**, 605–610 (1993).
20. Levett Sengers, J. M. H. in *Supercritical Fluids, Fundamentals for Application* (eds Kiran, E. & Levett Sengers, J. M. H.) 3–38 (NATO ASI Ser. E, 273, Kluwer, Dordrecht, 1994).
21. Sengers, J. V. & Levett Sengers, J. M. H. *Annu. Rev. Phys. Chem.* **37**, 189–222 (1986).
22. Iwasaki, H. & Takahashi, M. *J. Chem. Phys.* **74**, 1930–1943 (1981).
23. Debenedetti, P. G. & Reid, R. C. *Am. Inst. Chem. Eng. J.* **32**, 2034–2046 (1986).
24. Swaid, I. & Schneider, G. M. *Ber. Bunsenges. Phys. Chem.* **83**, 969–974 (1979).
25. Petsche, I. B. & Debenedetti, P. G. *J. Phys. Chem.* **95**, 386–399 (1991).
26. Kiran, E. & Levett Sengers, J. M. H. (eds) *Supercritical Fluids. Fundamentals for Application* 761–771 (NATO ASI Ser. E, 273, Kluwer, Dordrecht, 1994).
27. Brennecke, J. F., Tomasko, D. L., Peshkin, J. & Eckert, C. A. *Ind. Eng. Chem. Res.* **29**, 1682–1690 (1990).
28. Sun, Y.-P., Bennett, G., Johnston, K. P. & Fox, M. A. *J. Phys. Chem.* **96**, 1001–1007 (1992).
29. Ekart, M. P. et al. *Am. Inst. Chem. Eng. J.* **39**, 235–248 (1993).
30. Paulaitis, M. E., Krukonis, V. J. & Kurnik, R. T. *J. Phys. Chem.* **86**, 4948–4951 (1982).
31. Eckert, C. A., Ziger, D. H., Johnston, K. P. & Kim, S. J. *J. Phys. Chem.* **90**, 2738–2746 (1986).
32. Gates, J. A., Wood, R. H. & Quint, J. R. *J. Phys. Chem.* **86**, 4948–4951 (1982).
33. Wheeler, J. C. *Ber. Bunsenges. Phys. Chem.* **76**, 308–318 (1972).
34. Kurnik, R. T. & Reid, R. C. *Fluid Phase Equil.* **8**, 93–105 (1982).
35. Chialvo, A. A. *J. Phys. Chem.* **97**, 2740–2744 (1993).
36. Kwiatkowski, J., Lisicki, Z. & Majewski, W. *Ber. Bunsenges. Phys. Chem.* **88**, 865–875 (1984).
37. Dong, D.-C. & Winnick, M. A. *Can. J. Chem.* **62**, 2560–2565 (1984).
38. Knutson, B. L., Tomasko, D. L., Eckert, C. A., Debenedetti, P. G. & Chialvo, A. A. in *Supercritical Fluid Technology: Theoretical and Applied Approaches in Analytical Chemistry* (eds Bright, F. V. & McNally, M. E. P.) 60–72 (ACS Symp. Ser. 488, American Chemical Soc., Washington DC, 1992).
39. O'Brien, J. A., Randolph, T. W., Carlier, C. & Ganapathy, S. *Am. Inst. Chem. Eng. J.* **39**, 1061–1071 (1993).
40. Kim, S. & Johnston, K. P. *Am. Inst. Chem. Eng. J.* **33**, 1603–1611 (1987).
41. Yonker, C. R. & Smith, R. D. *J. Phys. Chem.* **92**, 2374–2378 (1988).
42. Phillips, D. J. & Brennecke, J. F. *Ind. Eng. Chem. Res.* **32**, 943–951 (1993).
43. Fulton, J. L., Yee, G. G. & Smith, R. D. *J. Am. Chem. Soc.* **113**, 8327–8334 (1991).
44. Gupta, R. M., Combes, J. R. & Johnston, K. P. *J. Phys. Chem.* **97**, 707–715 (1993).
45. Tomasko, D. L., Knutson, B. L., Pouillot, F., Liotta, C. L. & Eckert, C. A. *J. Phys. Chem.* **97**, 11823–11834 (1993).
46. Petsche, I. B. & Debenedetti, P. G. *J. Chem. Phys.* **91**, 7075–7084 (1989).
47. Chialvo, A. A. & Cummings, P. T. *Am. Inst. Chem. Eng. J.* **40**, 1558–1573 (1994).
48. Zhang, J., Lee, L. L. & Brennecke, J. F. *J. Phys. Chem.* **99**, 9268–9277 (1995).
49. Wu, R. S., Lee, L. L. & Cochran, H. D. *Ind. Eng. Chem. Res.* **29**, 977–988 (1990).
50. Tom, J. W. & Debenedetti, P. G. *Ind. Eng. Chem. Res.* **32**, 2118–2128 (1993).
51. McGuigan, D. B. & Monson, P. A. *Fluid Phase Equil.* **57**, 227–247 (1990).
52. Muñoz, F. & Chimowitz, E. H. *Fluid Phase Equil.* **71**, 237–272 (1992).
53. Cochran, H. D., Johnson, E. & Lee, L. L. *J. Supercrit. Fluids* **3**, 157–161 (1990).
54. Cummings, P. T., Chialvo, A. A. & Cochran, H. D. *Chem. Eng. Sci.* **49**, 2735–2748 (1994).
55. Cui, S. T. & Harris, J. G. *Chem. Eng. Sci.* **49**, 2749–2763 (1994).
56. Balbuena, P. B., Johnston, K. P. & Rossky, P. J. *J. Am. Chem. Soc.* **116**, 2689–2690 (1994).
57. Clifford, A. A. in *Supercritical Fluids. Fundamentals for Application* (eds Kiran, E. & Levett Sengers, J. M. H.) 449–479 (NATO ASI Ser. E, 273, Kluwer, Dordrecht, 1994).
58. Johnston, K. P. & Haynes, C. A. *Am. Inst. Chem. Eng. J.* **33**, 2017–2026 (1987).
59. Kim, S. & Johnston, K. P. *Chem. Eng. Commun.* **63**, 49–59 (1988).
60. Roberts, C. B., Chateaufneuf, J. E. & Brennecke, J. F. *J. Am. Chem. Soc.* **114**, 8455–8463 (1992).
61. Ellington, J. B., Park, K. M. & Brennecke, J. F. *Ind. Eng. Chem. Res.* **33**, 965–974 (1994).
62. Randolph, T. W. & Carlier, C. J. *Phys. Chem.* **96**, 5146–5151 (1992).
63. Zagrobelny, J. A. & Bright, F. V. *J. Am. Chem. Soc.* **114**, 7821–7826 (1992).
64. O'Shea, K. E., Combes, J. R., Fox, M. A. & Johnston, K. P. *Photochem. Photobiol.* **54**, 571–576 (1991).
65. Roberts, C. B., Zhang, J., Chateaufneuf, J. E. & Brennecke, J. F. *J. Phys. Chem.* **97**, 5618–5623 (1993).
66. Randolph, T. W., O'Brien, J. A. & Ganapathy, S. J. *Phys. Chem.* **98**, 4173–4179 (1994).
67. Kamat, S. V., Iwaszkewicz, B., Beckman, E. J. & Russell, A. J. *Proc. Natl. Acad. Sci. USA* **90**, 2940–2944 (1993).
68. Chaudhary, A. K., Beckman, E. J. & Russell, A. J. *J. Am. Chem. Soc.* **117**, 3728–3733 (1995).
69. Yeo, S.-D., Debenedetti, P. G., Radosz, M. & Schmidt, H.-W. *Macromolecules* **26**, 5207–5210 (1993).
70. Winters, M. A. et al. *J. Pharm. Sci.* **85**, 586–594 (1996).
71. Yeo, S.-D., Lim, G.-B., Debenedetti, P. G. & Bernstein, H. *Biotechnol. Bioeng.* **41**, 341–346 (1993).
72. Yeo, S.-D., Debenedetti, P. G., Patro, S. Y. & Przybicien, T. M. *J. Pharm. Sci.* **83**, 1651–1656 (1994).
73. Dixon, D. J., Johnston, K. P. & Bodemeier, R. A. *Am. Inst. Chem. Eng. J.* **39**, 127–139 (1993).
74. Tom, J. W. & Debenedetti, P. G. *J. Aerosol Sci.* **22**, 555–584 (1991).
75. Randolph, T. W., Randolph, A. D., Mebes, M. & Yeung, S. *Biotechnol. Prog.* **9**, 429–435 (1993).
76. Dooley, K. M., Kao, C.-P., Gambrell, R. P. & Knopf, F. C. *Ind. Eng. Chem. Res.* **26**, 2058–2062 (1987).
77. Macnaughton, S. J. & Foster, N. R. *Ind. Eng. Chem. Res.* **34**, 275–282 (1995).
78. Shaw, R. W., Brill, T. B., Clifford, A. A., Eckert, C. A. & Franck, E. U. *Chem. Eng. News* **69**(51), 26–39 (1991).
79. Jessop, P. G., Hsiao, T., Ikariya, T. & Noyori, R. *J. Am. Chem. Soc.* **118**, 344–345 (1996).
80. Beckman, E. J. *Science* **271**, 613–614 (1996).
81. Johnston, K. P. et al. *Science* **271**, 624–626 (1996).
82. Kazanian, S. G., Vincent, M. F., Bright, F. V., Liotta, C. L. & Eckert, C. A. *J. Am. Chem. Soc.* **118**, 1729–1736 (1996).

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# Extremely rapid bursts of TeV photons from the active galaxy Markarian 421

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**DISCRETE astronomical sources of photons in the TeV energy range are believed to be associated with regions in the relativistic outflow of particles and radiation from compact objects, such as neutron stars and black holes. The flux from such sources, together with the timescales on which they vary, can provide strong constraints on the emission mechanisms. Here we report the observation of two dramatic outbursts of TeV photons from the active galaxy Markarian 421 (Mrk421). In the first outburst, which had a doubling time of about one hour, the flux increased above the relatively quiescent value by more than a factor of 50, briefly making Mrk421 the brightest TeV source in the sky. In the second outburst, which lasted approximately 30 minutes, the flux increased by a factor of 20–25. These data suggest that the emission region is extremely small—perhaps even smaller than our Solar System. This could prove challenging for current theoretical models of such emissions.**

A camera consisting of 109 fast photomultipliers with a pixel size of  $0.25^\circ$  at the 10-metre reflector at the Fred Lawrence Whipple Observatory<sup>1,2</sup> images Cherenkov radiation from air showers. Image processing techniques refined using detailed Monte Carlo simulations<sup>2,4</sup> and frequent observations of the Crab Nebula over several years (ref. 5) enable discrimination of  $\gamma$ -ray-induced showers from the dominant background of showers created by high-energy cosmic rays. Four parameters that form the basis for selection criteria known as 'Supercuts' (ref. 3) are used to describe the approximately elliptical images: the angular width and length of the reduced image, the distance of the image centroid from the centre of the camera, and the orientation angle  $\alpha$  defined as the angle between a line from the centroid of the image to the centre of the camera and the major axis of the image ellipse. There is an excess number of events in the distribution of the angle  $\alpha$  near  $0^\circ$ , providing evidence for  $\gamma$ -rays. Background subtraction is determined from off-source runs at the same telescope elevation and from events with large  $\alpha$  angles in on-source data. A more thorough description of the methods used in the detection of  $\gamma$ -rays can be found in the literature<sup>3–5</sup>.

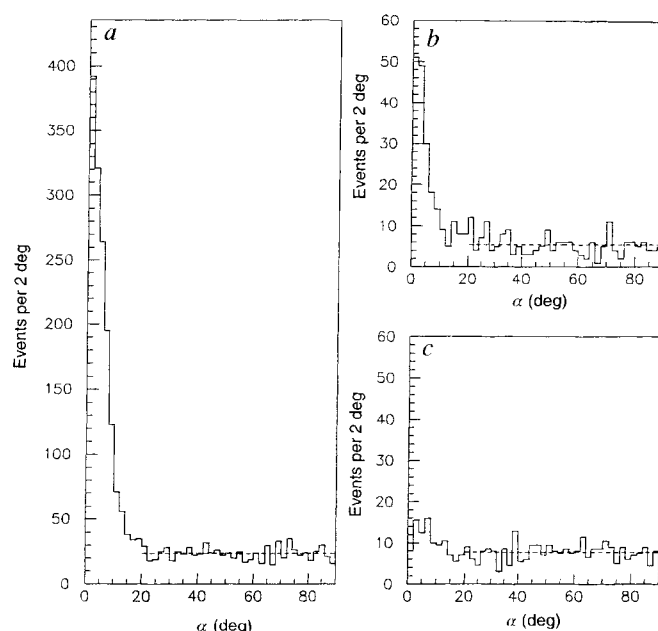


FIG. 1 Distributions in orientation angle  $\alpha$  for events that satisfy the Supercuts selections on image width, length and distance for events with more than 400 ADC counts. The signal region is  $\alpha < 15^\circ$ , and the broken line is the average level for  $\alpha > 20^\circ$ . *a*, Events in the large flare on 7 May 1996. *b*, Events in the 28-min run on 15 May 1996, during which the flare peaked. *c*, An average of events in the runs before and after the flare shown in *b*. In addition to the standard set of Supercuts, a new set of selection criteria for small events, which is currently under development, was used to analyse the flare below the size cut of 400 ADC counts where the background is significantly larger. The results indicated that the flare behaviour of the small events in the energy range below 350 GeV is also dramatic, reaching a rate of  $\sim 12.5$   $\gamma$ -rays per min. The possibility of spectral variations accompanying the giant flare was investigated by computing hardness ratios during the flare and comparing them to hardness ratios from data acquired during pre- and post-flare periods. The hardness ratio is based on the distribution of shower size (the ADC count in an event), a quantity that is approximately proportional to the energy of the incident  $\gamma$ -ray primary. Three intervals of shower size were used to search for spectral variability. No detectable spectral variations are attendant with the flare, and no spectral variations are observed within the flare event itself in this preliminary investigation.

The active galaxy Mrk421 is a very high energy (VHE)  $\gamma$ -ray source that is known to be variable on timescales of  $\geq 1$  day (refs 6, 7), and multi-wavelength observations have demonstrated a correlation between the VHE emission and emission at lower energies<sup>7–9</sup>. During routine monitoring of Mrk421 in the May 1996 observing period, the Whipple telescope recorded a dramatic flare, which produced the largest flux ever recorded from any source in the current VHE catalogue. The  $\alpha$  plot for the data from the night of 7 May 1996, selected by cuts on the first three image parameters, is displayed in Fig. 1*a*; a threshold cut of 400 analogue to digital converter (ADC) counts has been applied corresponding to an energy threshold of approximately 350 GeV. Events beyond an  $\alpha$  angle of  $20^\circ$  provide an estimate of the background contribution, which is very small for these data. We believe this to be the purest sample of VHE  $\gamma$ -rays ever recorded. A preliminary investigation of the spectrum reveals no large variations during the flare. The flux was observed to increase monotonically during the course of observation over a period of 2 h, beginning at a rate equal to the peak value of the highest previously observed flare<sup>8</sup>, and twice as high as any flare recorded during the 1995 observing season<sup>9</sup>. A counting rate of  $\sim 15$   $\gamma$ -rays per minute was reached, ten times as high as the steady rate observed from the Crab Nebula, which is a steady source for which  $\phi_{\text{CN}}$  is 1.5  $\gamma$ -rays per min above 350 GeV. The doubling time of this giant flare was of the order of



one hour during the observing session, which was terminated before moonrise. This change in rate is more than an order of magnitude faster than in previous data<sup>8,9</sup>. The temporal history of the observations is displayed in 9-minute intervals in Fig. 2a, where the  $\gamma$ -rate is determined from the excess events after background subtraction in the angular interval  $\alpha < 15^\circ$ . Follow-up observations on 8 May 1996 showed that Mrk421 had relaxed to a quiescent flux level of  $\sim 0.3 \phi_{\text{CN}}$ , suggesting that the decay timescale of the flare is probably shorter than one day. In its relatively quiescent state, the Mrk421 flux above 350 GeV is  $\sim 0.1\text{--}0.3 \phi_{\text{CN}}$ .

A second flare from Mrk421, remarkable for its very short duration, was observed eight days later. The temporal history of the observations in 4.5-minute intervals on the night of 15 May 1996 is displayed in Fig. 2b, where a rise and fall within a duration of much less than one hour is clearly evident, and a peak flux of 4–5 times the steady  $\phi_{\text{CN}}$  was reached. By relativistic causality, this astonishingly brief duration implies that the emission region is very compact. The doubling time of the flare event was  $< 15$  minutes, both for the rise and subsequent decline to the quiescent level. As in the case of the previous outburst, no large spectral variations are detectable during the flare, based on an analysis using hardness ratios. The  $\alpha$  plot for this flare is shown in Fig. 1b; the histogram corresponds to the 28-minute observation in which the flare peak occurred. In Fig. 1c the average of the on-source runs before and after is shown, including the tail of the flare. An off-source run had been taken just before the flare.

Production of TeV  $\gamma$ -rays in active galactic nuclei (AGNs), of which Mrk421 is an example, is poorly understood. It is generally assumed that most of the non-thermal emission derives from relativistic jets of material beamed towards the observer<sup>10</sup>. One class of popular models assumes that low-energy photons are boosted to high energies by inverse Compton scattering<sup>11–13</sup>. However, the large density of target photons required for this process creates a large optical depth through pair-production for the escape of TeV  $\gamma$ -rays. This problem can be circumvented, for example, if the site of inverse Compton scattering is located within the relativistic jet so that both target photons and boosted  $\gamma$ -rays are moving in the same direction with their luminosities modified by the Doppler factor  $\delta$  of the jet;  $\delta^{-1} = \gamma(1 - \beta \cos\theta)$ , where  $\beta$  and  $\gamma$  are the Lorentz factors of the jet, and  $\theta$  is the angle with respect to the observer<sup>14,15</sup>. If it is further assumed that flaring is correlated from optical to  $\gamma$ -ray wavelengths during this outburst, as has been observed for Mrk421 (refs 7, 9), and that the emission region in the jet is approximately spherical, a constraint on the Doppler factor is given<sup>9,15</sup> by flare duration time  $T$ ;  $\delta \geq 4.6 (T/10^5)^{-0.19}$ . For a duration of 30 minutes,  $\delta \geq 9$ , larger than values reported for variable AGNs detected by EGRET<sup>15</sup> in the GeV energy range. However, this value of  $\delta$  is consistent with a shift of the maximum luminosity of Mrk421 towards the TeV region, thereby making it a weaker source at EGRET energies, as is observed to be the case. Recent models<sup>16</sup> of the light curve for Mrk421 suggest that  $\delta \sim 10$  for a multi-wavelength description. This may be regarded as a lower limit in that considerations of detailed constraints on highly relativistic jets suggest<sup>17</sup> Lorentz factors of  $\gamma \sim 30\text{--}100$  are possible. For emission from a spherical blob with an observed time variability  $T$ , relativistic causality<sup>9,15</sup> requires the size of the emission region  $R$  in the jet to satisfy the inequality  $R < cT\delta/(1+z)$ , where  $z$  is the cosmological redshift;  $z = 0.03$  for Mrk421. With  $T \sim 1$  hour and  $\delta \sim 10$ , the spatial extent  $R$  is  $1 < R < 10$  light hours, a remarkably small region.

These observations place severe constraints on models of the production mechanism and site of high-energy  $\gamma$ -rays from AGNs. For example, the tightly constrained one-zone models mentioned above<sup>12,16</sup> are very sensitive to the dimension of the emission region  $R$  for an adequate description of the total energy spectrum of strong, variable  $\gamma$ -ray sources. These models used data from the EGRET observation<sup>18</sup> of variability in  $\gamma$ -ray emission of 3C279, in which the flux rose and fell within a period of approximately 12 days with a peak increase in flux of a factor of 4. There was some

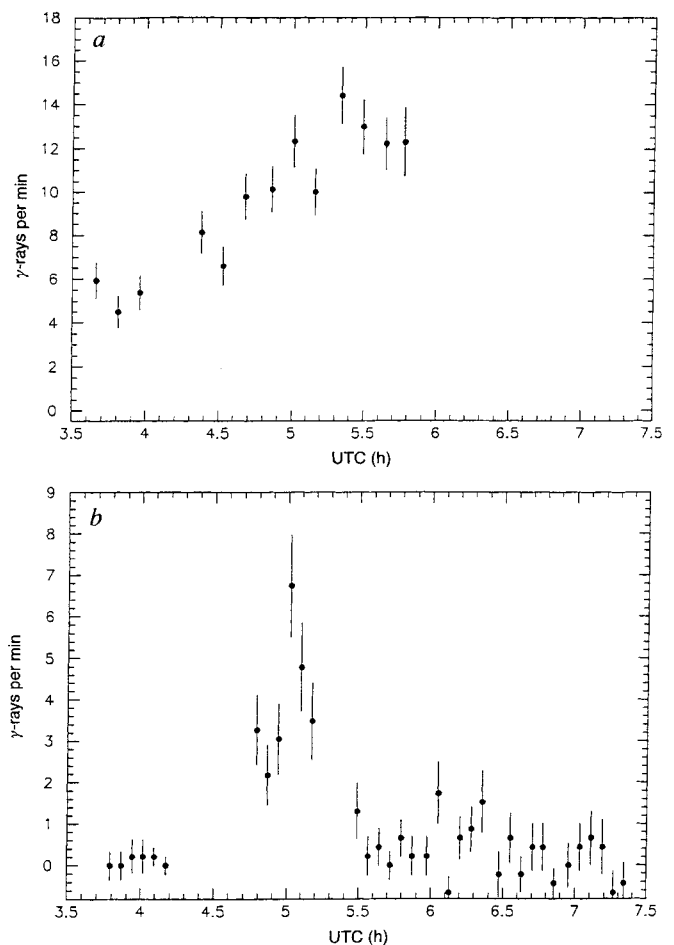


FIG. 2 Temporal histories of the two flare events. Rates are determined from the excess events after background subtractions in the interval  $\alpha < 15^\circ$ . The time axes are coordinated universal time (UTC) in hours. For the 7 May flare (a), each point is a 9-min integration; for the 15 May flare (b), the integration time is 4.5 min. The error bars are statistical standard deviations.

evidence for small fluctuations on timescales down to one day. In dramatic contrast, the VHE  $\gamma$ -ray flares from Mrk421 reported here exhibited a flux increase an order of magnitude greater than that of 3C279 in the first event, and a timescale more than two orders of magnitude shorter in the second.  $\square$

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1. Punch, M. et al. *Nature* **358**, 477–478 (1992).
2. Cawley, M. F. & Weekes, T. C. *Exp. Astron.* **6**, 7–42 (1995).
3. Reynolds, P. T. et al. *Astrophys. J.* **404**, 206–218 (1993).
4. Fegan, D. et al. in *Towards an Atmospheric Cherenkov Detector Vol. III* (ed. Kifune, T.) 149–162 (Universal Academy Press, Tokyo, 1994).
5. Weekes, T. C. et al. *AIP Conf. Proc.* **304**, 270–274 (1993).
6. Schubnell, M. S. et al. *Astrophys. J.* **460**, 644–650 (1996).
7. Macomb, D. J. et al. *Astrophys. J.* **449**, L99–L103 (1995).
8. Kerrick, A. D. et al. *Astrophys. J.* **438**, L59–L62 (1995).
9. Buckley, J. H. et al. *Astrophys. J.* (in the press).
10. Blandford, R. D. & Rees, M. J. In *Proc. Pittsburgh Conference on BL Lac Objects* (ed. Wolfe, A. N.) 328–347 (Univ. Pittsburgh Press, 1978).
11. Dermer, C. D. & Schlickeiser, R. *Astrophys. J.* **416**, 458–484 (1993).
12. Sikora, M., Begelman, M. C. & Rees, M. J. *Astrophys. J.* **421**, 153–162 (1994).
13. Königl, A. *Astrophys. J.* **243**, 700–709 (1981).
14. Blandford, R. D. & Königl, A. *Astrophys. J.* **232**, 34–48 (1979).
15. Mattox, J. R. et al. *Astrophys. J.* **410**, 609–614 (1993).
16. Inoue, S. & Takahara, F. *Astrophys. J.* **463**, 555–564 (1996).
17. Begelman, M. C., Rees, M. J. & Sikora, M. *Astrophys. J.* **429**, L57–L60 (1994).
18. Kniffen, D. A. et al. *Astrophys. J.* **411**, 133–136 (1993).

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# Hard elastic carbon thin films from linking of carbon nanoparticles

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**HARD carbon thin films find many technological applications—as protective or biocompatible coatings, for instance. A very hard and elastic form of carbon nitride, in which curved graphene sheets are interlinked owing to the presence of small amounts of nitrogen, has recently been reported<sup>1</sup>. The hardness of these films is thought to arise from the presence of  $sp^3$ -like bonds that introduce curvature into and bind together the  $sp^2$ -bonded graphitic planes, rather as they do in hard, highly tetrahedrally bonded amorphous carbon films<sup>2–4</sup>. Here we show that hard, elastic thin films of pure carbon can be created by depositing closed, hollow graphitic carbon nanoparticles—nanotubes<sup>5</sup> and carbon onions<sup>6</sup>—onto a substrate at high velocity. The particles are apparently disrupted on impact, causing them to link up. Electron-energy-loss spectra reveal a reduction in  $\pi$  ( $sp^2$ ) bonding in the intersecting regions of the nanoparticles, supporting the idea that they are covalently linked by tetrahedral  $sp^3$  bonds.**

Based on the high pressure arc plasma methods developed to produce fullerene molecules and nanotubes, we have used a high local gas pressure carbon arc technique for thin film deposition<sup>7</sup>. The carbon source is an arc on a graphite cathode within a high-pressure region created by a nitrogen or helium jet in the vicinity of the arc spot while keeping the pressure in the rest of the deposition chamber at 6 mtorr. Except for the gas jet, the apparatus is similar to vacuum arc systems used for deposition of highly tetrahedral amorphous carbon films<sup>2–4</sup>. The mechanism for nanotube formation by an arc, and the role of the local electric field, have been reported recently<sup>8</sup>. Nanotubes formed in the arcing process are carried by the high-pressure gas flow to the

low-pressure vacuum (6 mtorr) region where they, together with the carbon plasma, are condensed onto the substrate.

The films deposited on unheated substrates appear largely amorphous on initial examination in high-resolution electron microscopy (HREM). On closer inspection, well formed fullerene-like 'onion' structures<sup>6</sup> could be readily seen interspersed within an amorphous carbon matrix in the film; in a number of places, the fragmented (and disintegrated) nanoparticles were clustered together. Closed rings of planes, typical for nanotubes with their axes oriented parallel to the electron beam<sup>5</sup>, were also seen. Also present were discrete patches of approximately 1  $\mu$ m diameter made up of larger nanotubes interspersed with more complex fullerene structures. Such a region is shown in Fig. 1a. The results in Fig. 1 suggest that it is possible to form dense nanotube regions within a thin film.

The formation of dense nanotube regions is not in itself sufficient to obtain the cross-linking of curved graphene planes. Closer examination of a dense nanotube region suggests that many nanotubes split on impact, and that linking of planes in adjacent tubes can occur. The splitting of planes in the tube wall is clearly seen in Fig. 1b, an enlargement of a sub-region in Fig. 1a, where the set of curving planes at the end of the large tube separate. The separated outer planes in turn appear to buckle away from the tube, and link together. In other regions of Fig. 1, tube and distorted graphene fragments in close proximity, appearing to intersect in projection, can be seen. Nanotubes very similar to those in Fig. 1 were also seen in films deposited in the presence of a He jet at the arc surface. It is, however, difficult to ascertain from the HREM images alone the nature of the linked regions. Electron-energy-loss spectroscopy (EELS) was also used to gain more information on the bonding in the regions where tubes and graphene planes lie in close proximity.

Trace a in Fig. 2 is an EEL spectrum that was obtained from an amorphous region where no plane-like structure was discerned. The two characteristic carbon features seen in EELS, the  $1s - \pi^*$  (285 eV) and  $1s - \sigma^*$  (296 eV) loss edges are marked. By taking the ratio of the integral under the two peaks and normalizing it to that for graphite, it is possible to quantify the relative amount of  $\pi$  bonding present in the films<sup>3,9</sup>. Spectrum a in Fig. 2 is typical for that measured for highly diamond-like 'tetrahedral amorphous carbon films with  $sp^2/sp^3$  ratios of 0.2–0.3. The EELS features around 400 eV are due to nitrogen and show that N from the high-pressure gas jet at the arc has also been incorporated in the films.

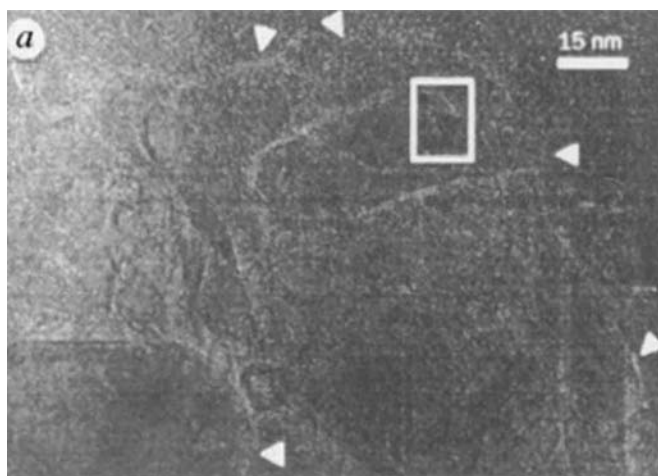


FIG. 1 High-resolution electron micrographs, obtained with a JEOL 2000EXTEM instrument. The film was deposited on an Si[100] substrate maintained at room temperature and placed 25 cm in front of the arc spot. The arc was struck in high-pressure nitrogen. The films were prepared for HREM by dissolving the Si substrate in  $\text{HF:HNO}_3\text{:H}_2\text{O}$  solution and lifting the

films off onto Cu grids. a, An area of densely packed nanotubes. Several of the largest nanotube fragments are indicated by arrows. b, Enlarged area from the region marked in a. The set of planes (marked '1') forming a tube wall separates at two points (arrows). After splitting, some planes (marked '2') buckle away from the tube.

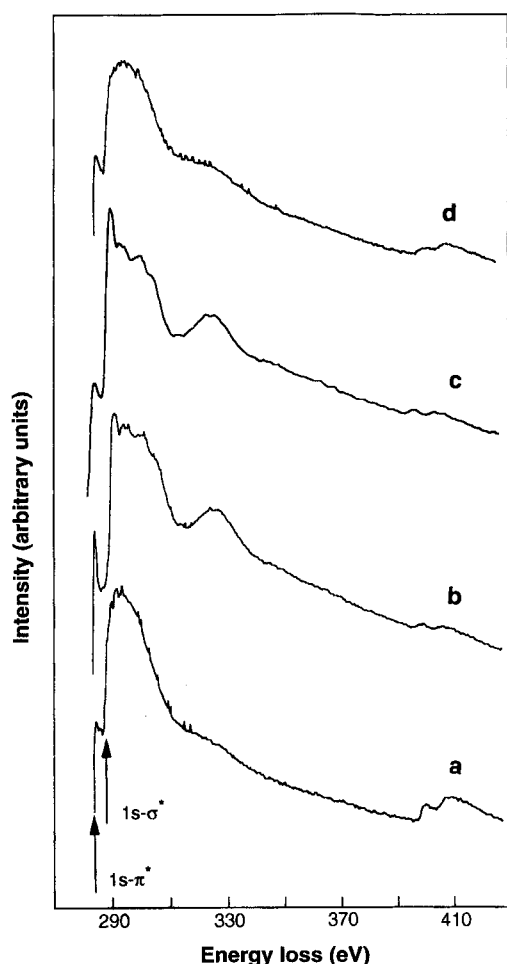


FIG. 2 The bonding of the films was examined by analysing the carbon K-edge fine structure derived from electron-energy-loss spectroscopy (EELS). Carbon K-edge spectra obtained from several regions of the film deposited at RT are shown: trace a, from an amorphous region; trace b, from a wall region in a nanotube; trace c, from a region where two sets of graphene planes interlink. Trace d was obtained from a film deposited at 350 °C. The axes of the graphene planes were normal to the electron probe beam in traces b and c. The EELS was carried out using a 1-nm-diameter electron probe in a VG HB601 scanning transmission electron microscope with a Gatan 666 parallel detector having an energy resolution of 0.5–1 eV. The primary beam energy was 100 keV with a convergence angle of 10.6 mrad; the EELS collection angle for the inelastically scattered electrons was 6.8 mrad.

The N content in the amorphous region, estimated by the relative intensities of the C and N peaks and their respective interaction cross-sections, was 16%. Spectrum b in Fig. 2 was measured over an area of 1 nm from a tube wall region (with the tube axis in the plane of the film) which shows the distinct graphite-like plane structure (Fig. 1a). This spectrum is typical of graphite, showing sharp features with a very distinct  $1s - \pi^*$  loss peak. The N feature is very faint and probably originates from amorphous regions underneath the extended graphite plane region.

The EEL spectrum c in Fig. 2 was obtained from a very thin (20 nm) region in which a nanotube, lying with its axis parallel to the beam, apparently intersects a set of graphite planes with their  $c$ -axes lying at right angles to the nanotube axis. The  $1s - \pi^*$  peak in spectrum c is much less intense with respect to the  $1s - \sigma^*$  than that in spectrum b, and also lacks the sharpness typical of a crystalline feature. The  $1s - \sigma^*$  loss peak, however, retains the sharp feature typical of a crystalline region and has a much higher

TABLE 1 Properties of nanoparticle carbon films

| Film                         | Hardness (GPa) | Elastic recovery (%) |
|------------------------------|----------------|----------------------|
| NC (RT, N <sub>2</sub> )     | 38             | 55                   |
| NC (RT, He)                  | 40             | 40                   |
| NC (350 °C, N <sub>2</sub> ) | 56             | 85                   |
| NC (350 °C, He)              | 53             | 80                   |
| ta-C                         | 50             | 30                   |

The first column gives the deposition temperature and gas used to prepare the films: NC, nanoparticle carbon; ta-C, tetrahedral amorphous carbon; RT, room temperature. Note that the elastic recovery of the NC films deposited at 350 °C is far greater than that of the ta-C, though hardness values are similar.

relative intensity. When the 1-nm electron probe was moved away from the intersecting region, to either the graphene planes of the nanotube or the graphite planes, the EEL spectrum shows a sharp  $1s - \pi^*$  crystalline loss peak as in spectrum b. The reduced intensity of  $1s - \pi^*$  loss peak relative to the  $1s - \sigma^*$  peak in spectrum c can be interpreted as a reduction of  $\pi$  bonds in the intersecting region compared to the isolated tube and graphite regions. The reduction of  $\pi$  bonds may be taken as the basis for the supposition that the carbon planes in the intersecting region exhibit some four-fold co-ordinated bonding, perhaps similar to the hexagonal diamond structure. More detailed examination and analysis of the bonding in these regions is needed, however, before any firm conclusions can be drawn.

The results presented here show that irreversible splitting of nanotubes, together with buckling and distortion<sup>10,11</sup>, can take place on impact. The splitting process may commence when an open tube strikes (at high energy) a substrate with its axis normal to the surface. If the impact takes place on existing nanotube fragments in the growing film, the release of energy may be sufficient for interlinking of the graphite tube planes. When a film was deposited under the same arc plasma conditions but with the substrate heated to 350 °C, distinct structure as in Fig. 1 could

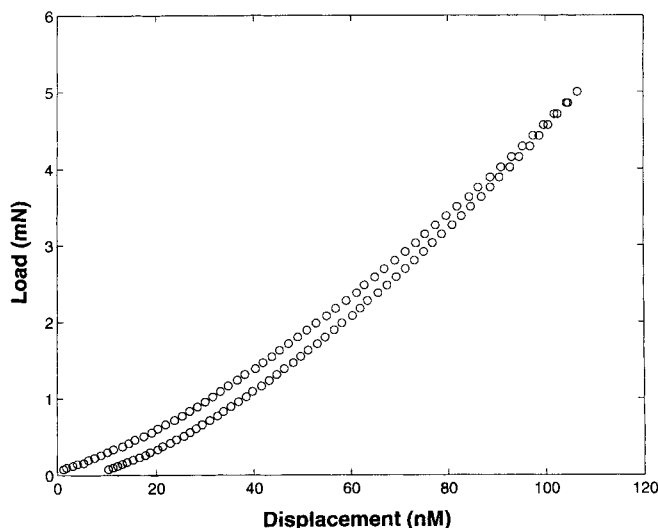


FIG. 3 Typical load-displacement curve measured for a 500-nm-thick nanoparticle carbon film deposited at 350 °C. The measurement was done using ultra-low-load Fisher micro-indenter with a Vickers diamond tip. The hardness was calculated using the plastic displacement obtained from the intercept with the displacement axis of the gradient of the unloading curve at maximum load (5 mN). This procedure was calibrated by evaluating materials with known hardness at maximum load (5 mN) and the gradient of the curve. The elastic recovery was determined from the residual displacement (10 nm) and the maximum displacement.



not be detected. We therefore infer that when the substrate is heated, the nanotube and onion structures disintegrate on impact far more efficiently and to a greater extent.

Tetrahedral amorphous carbon has been the hardest form of non-diamond carbon thin film obtained to date. The hardness and elastic recovery measured from indentation curves (with a 5 mN maximum loading) on similar-thickness films—500 nm—of nanoparticle carbon (NC) and tetrahedral amorphous carbon are shown in Table 1. A typical indentation characteristic of an NC film deposited at 350 °C is shown in Fig. 3. The absence of large nanotube regions in this film, compared to the films deposited at room temperature, and the fact that it has a high hardness and elastic recovery, suggest that a qualitatively new form of carbon thin film is obtained.

Considering the difference between the NC films deposited at

room temperature and high temperature in terms of mechanical properties, it is possible that breakup of the large nanotube regions results in a denser structure without the weak interplanar coupling of the curved hexagonal planes. It seems reasonable to suppose that by heating the substrate the C atoms become more mobile, and thus interlinking becomes more prevalent as the tubes break up into smaller fragments. In the resulting film, fine structure would not be easily distinguishable from the surrounding amorphous matrix (spectrum d in Fig. 2). Although insight into the detailed structure of superhard and superelastic nanoparticle carbon nanoparticle carbon films requires further experimental and theoretical study, their relative ease of deposition makes them particularly suitable for evaluation as protective coatings in applications ranging from computer memory disks to surgical implants. □

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1. Sjöström, H., Stafström, S., Roman, M. & Sundgren, J.-F. *Phys. Rev. Lett.* **75**, 1336–1339 (1995).
2. McKenzie, D. R., Müller, D. & Pailthorpe, B. A. *Phys. Rev. Lett.* **67**, 773–776 (1991).
3. Fallon, P. J. et al. *Phys. Rev. B* **48**, 4777–4782 (1993).
4. Charrier, C. et al. *Diamond Relat. Mater.* **3**, 41–46 (1994).
5. Iijima, S. *Nature* **354**, 56–57 (1991).
6. Ugarte, D. *Nature* **359**, 707–709 (1992).
7. Rogozin, A. & Fontana, R. Paper presented at *Int. Congr. Thin Films & Metallurgical Coatings*, San Diego, April, 1996.

8. Gamaly, E. G. & Ebbesen, T. W. *Phys. Rev. B* **52**, 2083–2089 (1995).
9. Berger, S. D., McKenzie, D. R. & Martin, P. J. *Phil. Mag. Lett.* **57**, 285–288 (1988).
10. Despres, J. F., Daguerre, E. & Lafdi, K. *Carbon* **33**, 87–92 (1995).
11. Iijima, S., Brabec, C., Maiti, A. & Bernholc, J. J. *Chem. Phys.* **104**, 2089–2092 (1996).

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## Scaling behaviour of heartbeat intervals obtained by wavelet-based time-series analysis

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**BIOLOGICAL time-series analysis is used to identify hidden dynamical patterns which could yield important insights into underlying physiological mechanisms. Such analysis is complicated by the fact that biological signals are typically both highly irregular and non-stationary, that is, their statistical character changes slowly or intermittently as a result of variations in background influences<sup>1–3</sup>. Previous statistical analyses of heartbeat dynamics<sup>4–6</sup> have identified long-range correlations and power-law scaling in the normal heartbeat, but not the phase interactions between the different frequency components of the signal. Here we introduce a new approach, based on the wavelet transform and an analytic signal approach, which can characterize non-stationary behaviour and elucidate such phase interactions. We find that, when suitably rescaled, the distributions of the variations in the beat-to-beat intervals for all healthy subjects are described by a single function stable over a wide range of timescales. However, a similar scaling function does not exist for a group with cardiopulmonary instability caused by sleep apnoea. We attribute the functional form of the scaling observed in the healthy subjects to underlying nonlinear dynamics, which seem to be essential to normal heart function. The approach introduced**

**here should be useful in the analysis of other nonstationary biological signals.**

A random process is stationary if its statistical characteristics are invariant under time shifts, that is, if they remain the same when  $t$  is replaced by  $t + \Delta$ , where  $\Delta$  is arbitrary. The probability densities, together with the moment and correlation functions, do not then depend on the absolute position of the points on the time axis, but only on their relative configuration. Non-stationarity, an important aspect of biological variability, can be associated with patterns of different drifts in the mean value of a given signal, or with changes in its variance which may be gradual or abrupt. Time series of beat-to-beat (R–R) heart-rate intervals (Fig. 1a), obtained from digitized electrocardiograms, are known to be non-stationary and exhibit extremely complex behaviour<sup>7</sup>. A typical feature of such non-stationary signals is the presence of ‘patchy’ patterns that change over time (Fig. 1b). Heterogeneous properties may be even more strongly expressed in certain cases of abnormal heart activity. Traditional approaches (such as the power spectrum and correlation analysis<sup>4,8,9</sup>) are not suited for such non-stationary (patchy) sequences, nor do they carry information stored in the Fourier phases which is crucial for determining nonlinear characteristics.

To address these problems, we present an alternative method, which we call ‘cumulative variation amplitude analysis’, to study the subtle structure of physiological time series. This method involves the sequential application of a set of algorithms based on wavelet and Hilbert transform analysis. First, we apply the wavelet transform (Fig. 1c), because it does not require stationarity and preserves the Fourier phase information. The wavelet transform<sup>10–12</sup> of a time series  $s(t)$  is defined as

$$T_{\psi}(t_0, a) \equiv \frac{1}{a} \int_{-\infty}^{\infty} s(t) \psi\left(\frac{t-t_0}{a}\right) dt \quad (1)$$

where the analysing wavelet  $\psi$  has a width of the order of the scale  $a$ , and is centred at time  $t_0$ . The wavelet transform  $T$  is sometimes called a ‘mathematical microscope’ because it allows the study of properties of the signal on any chosen scale  $a$ . For high frequencies (small  $a$ ), the  $\psi$  functions have good localization (being effectively non-zero only on small sub-intervals), so short-time regimes or high-frequency components can be detected by the wavelet analysis. However, a wavelet with too large a value of scale

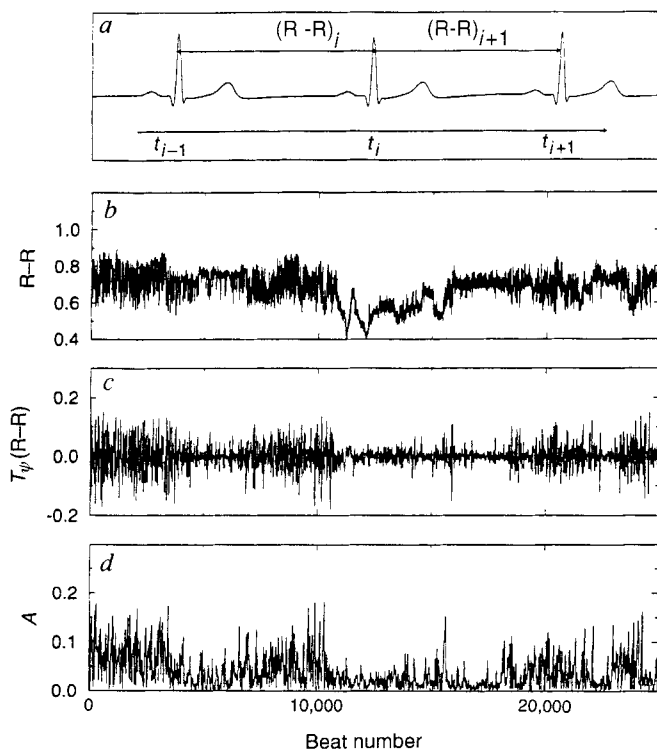


FIG. 1 *a*, Segment of electrocardiogram showing beat-to-beat ( $R-R$ ) intervals. *b*, Plot of  $R-R$  time series against consecutive beat number for a period of 6 h ( $\sim 2.5 \times 10^4$  beats). Non-stationarity (patchiness) is evident over both long and short timescales. Although these patches clearly differ in the amplitude and frequency of variations, their quantitative characterization remains an open problem. *c*, Wavelet transform  $T_\psi(R-R)$  of the  $R-R$  signal in *b*. Non-stationarities related to constants and linear trends have been filtered. The first derivative of the gaussian  $\psi^{(1)}$  is orthogonal to segments of the time series with different constant local average. This results in fluctuations of the wavelet transform values around zero, with highest spikes at the positions where a sharp transition in the constant value in the constant value occurs. Thus the large spikes indicate the boundaries between patterns with different local average in the signal, and the smaller fluctuations represent variations of the signal within a given dynamical regime. Because  $\psi^{(1)}$  is not orthogonal to linear (non-constant) trends, the presence of consecutive linear trends in the  $R-R$  intervals will give rise to fluctuations of the wavelet transform values around different non-zero levels corresponding to the slopes of the linear trends.  $\psi^{(2)}$  and higher order derivatives can eliminate the influence of linear as well as nonlinear trends in the fluctuations of the wavelet transform values. *d*, Instantaneous amplitudes  $A(t)$  of the wavelet transform signal in *c*;  $A(t)$ , calculated using the Hilbert transform, measures the cumulative variations in the interbeat intervals over an interval proportional to the wavelet scale  $a$ .

$a$  (low frequency) will filter out almost the entire frequency content of the time series, thus losing information about the intrinsic dynamics of the system. We focus our 'microscope' on scale  $a = 8$  beats, which smoothes locally very high-frequency variations, and best probes patterns of specific duration (30 s to 1 min) (see Fig. 2 legend). The wavelet transform can eliminate local polynomial behaviour (trends) in the non-stationary signal by an appropriate choice of the analysing wavelet  $\psi$  (ref. 13). In our study we use derivatives of the gaussian function:  $\psi^{(n)} \equiv d^n/dt^n e^{-\frac{1}{2}t^2}$ .

The wavelet transform is thus a cumulative measure of the variations in the heart-rate signal over a region proportional to the wavelet scale, so study of the behaviour of the wavelet values can reveal intrinsic properties of the dynamics that are masked by non-stationarity.

The next step of the cumulative variation amplitude analysis is to extract the amplitudes of the variations in the beat-to-beat signal, by means of an analytic signal approach<sup>8,14</sup> that also does not require stationarity. Let  $s(t)$  represent an arbitrary signal. The analytic signal, a complex function of time, is defined by  $S(t) \equiv$

$s(t) + i\tilde{s}(t) = A(t)e^{i\phi(t)}$ , where  $\tilde{s}(t)$  is the Hilbert transform<sup>15</sup> of  $s(t)$ ,  $A(t) \equiv \sqrt{s^2(t) + \tilde{s}^2(t)}$  is the amplitude, and  $\phi(t) \equiv \tan^{-1}(\tilde{s}(t)/s(t))$  is the phase.

We studied the distribution of the amplitudes of the beat-to-beat variations (Fig. 1*d*) for a group of healthy subjects ( $N = 18$ , 5 males and 13 females; age, 20–50 years, mean 34 years) and a group of subjects<sup>16</sup> with obstructive sleep apnoea<sup>17</sup> ( $N = 16$  males; age, 32–56 years, mean 43 years). To minimize non-stationarity resulting from changes in the level of activity, we begin by considering night-phase (0:00 to 6:00) records of interbeat intervals ( $\sim 10^4$  beats) for both groups. Inspection of the distribution functions of the amplitudes of the cumulative variations indicates marked differences between individuals (Fig. 2*a*). These differences are not surprising, given the underlying physiological differences among healthy subjects.

We next analyse the distributions of the beat-to-beat variation amplitudes. For the healthy group, these are well fit by the generalized homogeneous form<sup>18</sup> (the gamma distribution)

$$P(x, b) = \frac{b^{v+1}}{\Gamma(v+1)} x^v e^{-bx} \quad (2)$$

where  $b \equiv v/x_0$ ,  $\Gamma(v+1)$  is the gamma function,  $x_0$  is the position of the peak  $P = P_{\max}$ , and  $v$  is the fitting parameter (Fig. 3*a*). A function  $P(x, b)$  is a generalized homogeneous function if there exist two numbers  $\alpha$  and  $\beta$ , called scaling powers, such that, for all positive values of the parameter  $\lambda$ ,

$$P(\lambda^\alpha x, \lambda^\beta b) = \lambda P(x, b) \quad (3)$$

Generalized homogeneous functions are defined as solutions of this functional equation;  $P(x, b)$  satisfies this equation with  $\alpha = -1$  and  $\beta = 1$ .

Functions describing physical systems near their critical points are generalized homogeneous functions<sup>19</sup>. Data collapse is one of the important properties of generalized homogeneous functions: instead of data points falling on a family of curves, one for each value of  $b$ , data points collapse onto a single curve given by the scaling function

$$\tilde{P}(u) \equiv P(x, b)/b \quad (4)$$

where the number of independent variables is reduced by defining the scaled variable  $u \equiv bx$ . Our results show that a common scaling function  $\tilde{P}(u)$  defines the probability density of the magnitudes of the variations in the beat-to-beat intervals for each healthy subject. Note that it is sufficient to specify only one parameter  $b$  to characterize the heterogeneous heartbeat variations for each subject in this group.

To test the hypothesis that there is a hidden, possibly universal, structure to these heterogeneous time series, we rescale the distributions and find for all healthy subjects that the data conform to a single scaled plot ('data collapse') (Fig. 2*b*). Such behaviour is reminiscent of a wide class of well-studied physical systems with universal scaling properties<sup>19,20</sup>. In contrast, the subjects with sleep apnoea show individual probability distributions (Fig. 2*c*) which fail to collapse (Fig. 2*d*).

We also analysed heart-rate dynamics for the healthy subjects during daytime hours (12:00 to 18:00). Our results indicate that the observed, apparently universal behaviour holds not only for the night phase but also for the day phase (Fig. 3*b*). Moreover, we find the observed scaling to be stable for a wide range of timescales (Fig. 3*c*).

To ascertain whether the observed scaling of the distributions for healthy subjects is an intrinsic property of normal heartbeat dynamics, we test the cumulative variation amplitude method on artificially generated signals with known properties. Our analysis of uniformly distributed random numbers in the interval  $[0, 1]$  and of gaussian-distributed noise with and without long-range power-law correlations shows that, after the wavelet transform, the amplitude distributions follow the Rayleigh probability distribution  $(x/\sigma^2)e^{-x^2/\sigma^2}$ . This finding agrees with the central limit theorem,

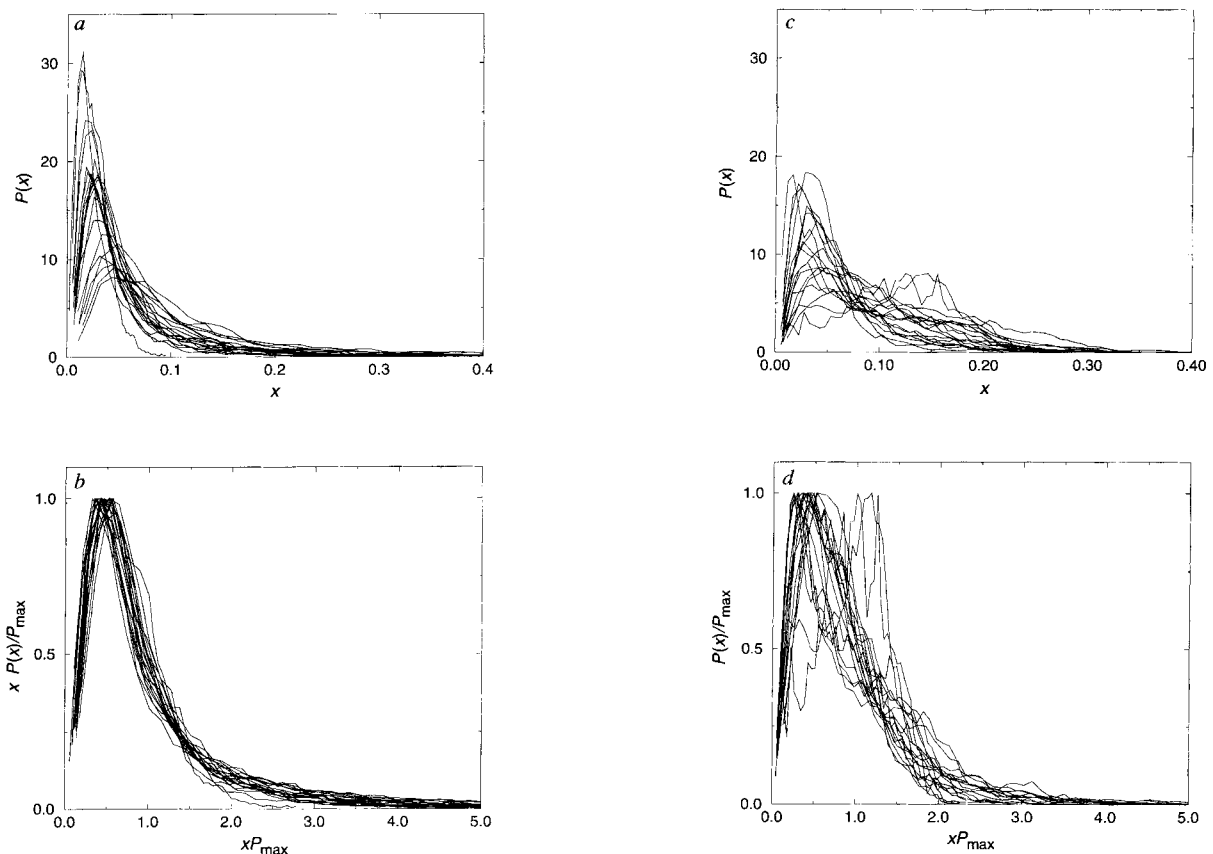


FIG. 2 *a*, Probability distributions  $P(x)$  of the amplitudes of heart-rate variations  $x \equiv A(t)$  for a group of 18 healthy adults. Individual differences are indicated by the different average value and widths (standard deviations) of these distributions. All distributions are normalized to unit area. *b*, Same probability distributions as *a*, after rescaling  $P(x)$  by  $P_{\max}$  and  $x$  by  $1/P_{\max}$  to preserve the normalization to unit area. This rescaling is equivalent to that discussed in the text (equation (4)), as  $P(x) \equiv P(x, b)$  and  $P_{\max} \propto b$ . The data points collapse onto a single curve. *c*, Probability distributions for a group of 16 subjects with obstructive sleep apnoea. The second (right-hand) peak in the distributions for the sleep-apnoea subjects corresponds to the transient emergence of characteristic pathological oscillations in the heart rate associated with periodic breathing<sup>6,17</sup>. *d*, Distributions for the apnoea group after the same rescaling as in *b*. The absence of data collapse demonstrates deviation from normal heart behaviour. Direct analysis of interbeat interval histograms does not lead to data collapse or separation between the healthy and apnoea group.

which can be expressed as a property of convolutions (in our case wavelet transform): the convolution of a large number of positive functions is approximately a gaussian function, and the instantaneous amplitudes of a gaussian process follow the Rayleigh probability distribution<sup>21</sup>.

We perform parallel analysis on surrogate data obtained from a healthy subject by Fourier transforming the original time series, preserving the amplitudes of the Fourier transform but randomizing the phases, and performing an inverse Fourier transform (Fig. 4). Thus both the original and surrogate signals have identical power spectra. Application of the cumulative variation amplitude analysis on this surrogate signal results again in a Rayleigh distribution, whereas the original time series has a distribution with an exponential tail. This test clearly indicates the important role of phase correlations in the R–R time series. The presence of these correlations is probably related to the underlying nonlinear dynamics. A characteristic feature of nonlinear (as opposed to linear) systems is the coupling of their components. These mode interactions may lead to non-random phase structure, and, in the context of the heartbeat, may account for the visually ‘patchy’ appearance of the normal time series. Our procedure, by preser-

Moreover, the direct application of the Hilbert transform yielding the probability distribution of the instantaneous amplitudes of the original signal does not distinguish clearly healthy from abnormal cardiac dynamics. Hence the crucial feature of the wavelet transform is that it extracts dynamical properties hidden in the cumulative variations. For the healthy group, good data collapse is observed with a stable scaling form for wavelet scales  $a = 2$  up to  $a = 2$  up to  $a = 64$ . However, for very small scales ( $a = 1, 2$ ), the average of the rescaled distributions of the apnoea group is indistinguishable from the average of the rescaled distributions of the healthy group. Hence very high frequencies are equally present in the signals from both groups. Our analysis yields the most robust results when  $a$  is tuned to probe the collective properties of patterns with a duration of 30 s to 1 min in the time series ( $a = 8, 10$ ). The subtle difference in the tail of the distributions between day and night phases is also best seen for this scale range (Fig. 3).

ving the collective phase properties of the original signal which cannot be detected by conventional power-spectrum analysis, uncovers a previously unknown nonlinear feature of healthy heart-rate fluctuations.

Furthermore, our finding suggests that, for healthy individuals, there may be a common structure to this nonlinear phase interaction. This scaling property cannot be explained by activity, as we analysed data from subjects during nocturnal hours, or by sleep stage transitions, as we found a similar pattern during daytime hours. The basis of this robust temporal structure remains unknown and presents a new challenge to understanding nonlinear mechanisms of heartbeat control. We also find that subjects with sleep apnoea, a common and important instability of cardio-pulmonary regulation, show a dramatic alteration in the scaling pattern that may be related to pathologic mode locking associated with periodic breathing dynamics<sup>22</sup>.

We believe that the method developed here can pick up differences that are missed by other approaches for two reasons: it can ‘filter out’ dominant features related to non-stationarities and thereby become sensitive to hidden scaling features; and is sensitive to the time ordering of events (provided a sensible choice



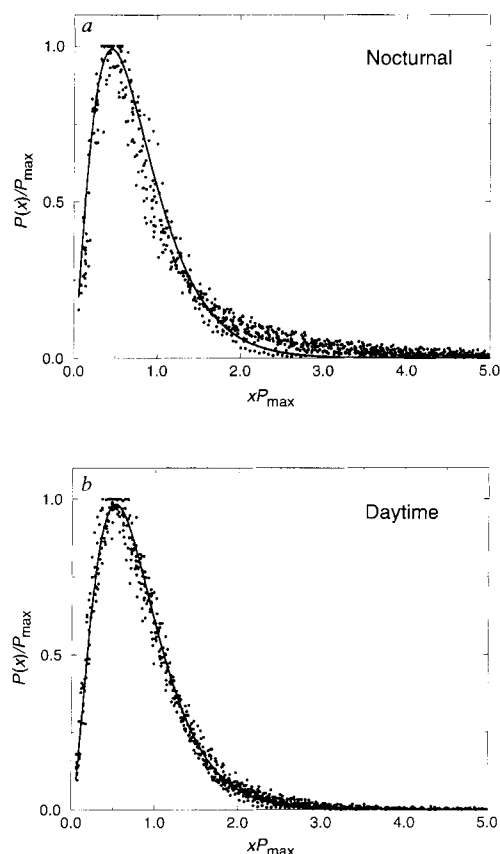
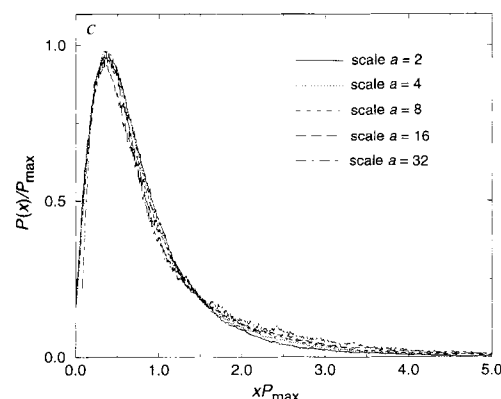


FIG. 3 *a*, The solid line is a fit of the rescaled distributions of the beat-to-beat variation amplitudes of the 18 healthy subjects during sleep hours to a



stable gamma distribution with  $\nu = 1.4 \pm 0.1$  (note that the stable gamma form has been used previously in the literature to describe other processes, such as the spike activity of a single neuron in ref. 23). *b*, Data for 6-h records of R-R intervals for the day phase of the same control group of 18 healthy subjects demonstrate similar scaling behaviour with a gamma distribution and  $\nu = 1.8 \pm 0.1$ , showing that the observed common structure for the healthy heart dynamics is not confined to the nocturnal phase. Semilog plots of the averaged distributions show a systematic deviation from the exponential form (slower decay) in the tails of the night-phase distributions, whereas the day-phase distributions follow the exponential form over almost the entire range. The tail of the observed distribution for the night phase indicates higher probability of larger variations in the healthy heart dynamics during sleep hours in comparison with the daytime dynamics. The maximum difference between the cumulative distributions of the individual subjects and the gamma fit in *a*, evaluated using the Kolmogorov-Smirnov test, can be a good index to separate the healthy from the apnoea group. Analysis of the mean and the variance of the individual distributions also shows clear separation for both groups. *c*, Average of the rescaled distributions for the amplitudes of the cumulative variations for the healthy group during nocturnal hours. Note that the observed gamma scaling is stable for a wide range of the wavelet transform scales *a*.

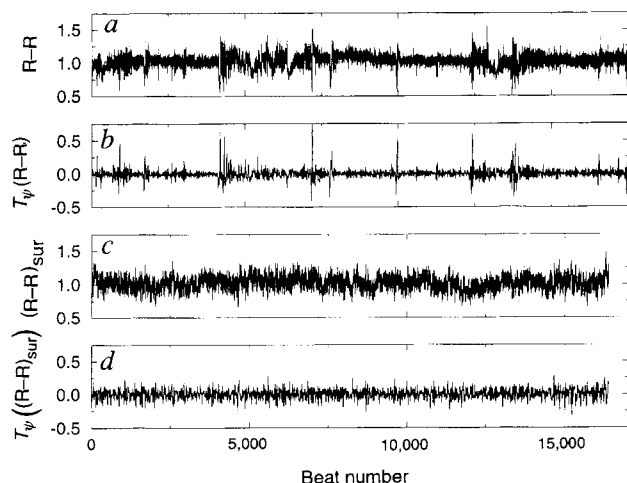


FIG. 4 *a*, Original R-R time series as a function of beat number. *b*, Wavelet transform  $T_\psi(R-R)$  of this series. *c*, Surrogate  $(R-R)_{sur}$  signal after phase randomization. *d*, Wavelet transform of the surrogate signal which is more homogeneous (less patchy) than in *b*. *e*, Probability distributions of the

amplitudes of variations after wavelet transform of the original and surrogate signals, as well as the theoretical Rayleigh distribution. The theoretical Rayleigh agrees with the distribution of the wavelet transform of the surrogate signal with randomized phases.

is made for the scale parameter *a*). Thus the dual use of wavelet and Hilbert transform techniques may be of practical diagnostic and prognostic value, and can be applied to a wide range of heterogeneous, 'real world' physiological signals.  $\square$

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1. Box, G. E. P., Jenkins, G. M. & Reinsel, G. C. *Time Series Analysis: Forecasting and Control* (Prentice-Hall, Englewood Cliffs, NJ, 1994).

- Shlesinger, M. F. *Ann. NY Acad. Sci.* **504**, 214–228 (1987).
- Liebovitch, L. S. *Biophys. J.* **55**, 373–377 (1989).
- Bassingthwaite, J. B., Liebovitch, L. S. & West, B. J. *Fractal Physiology* (Oxford Univ. Press, New York, 1994).
- Peng, C.-K., Havlin, S., Stanley, H. E. & Goldberger, A. L. *Chaos* **5**, 82–87 (1995).
- Aghili, A. A., Rizwan-uddin, M., Griggin, P. & Moorman, J. R. *Phys. Rev. Lett.* **74**, 1254–1257 (1995).
- Kitney, R. I., Linkens, D., Selman, A. C. & McDonald, A. H. *Automedica* **4**, 141–153 (1982).
- Panter, D. *Modulation, Noise and Spectral Analysis* (McGraw-Hill, New York, 1965).
- Akselrod, S. et al. *Science* **213**, 220–222 (1981).
- Grossmann, A. & Morlet, J. *Mathematics and Physics, Lectures on Recent Results* (World Scientific, Singapore, 1985).

11. Daubechies, I. *Comments Pure Appl. Math.* **41**, 909–996 (1988).
12. Muzy, J. F., Bacry, E. & Arneodo, A. *Int. J. Bifurc. Chaos* **4**, 245–302 (1994).
13. Arneodo, A., Bacry, E., Graves, P. V. & Muzy, J. F. *Phys. Rev. Lett.* **74**, 3293–3296 (1995).
14. Vainshtein, L. A. & Vakman, D. E. *Separation of Frequencies in the Theory of Oscillations and Waves* (Nauka, Moscow, 1983).
15. Gabor, D. J. *Inst. Elect. Eng.* **93**, 429–457 (1946).
16. MIT-BIH Polysomnographic Database CD-ROM 2nd edn (MIT-BIH Database Distribution, Cambridge, 1992).
17. Guilleminault, C., Connolly, S., Winkle, R., Melvin, K. & Tilkian, A. *Lancet* **(i)**, 126–131 (1984).
18. Stauffer, D. & Stanley, H. E. *From Newton to Mandelbrot: A Primer in Theoretical Physics* 2nd edn (Springer, Heidelberg, 1996).
19. Barabási, A.-L. & Stanley, H. E. *Fractal Concepts in Surface Growth* (Cambridge Univ. Press, 1995).
20. Kertész, J. & Vicsek, T. *Fractals in Science* (eds Bunde, A. & Havlin, S., (Springer, Heidelberg, 1994).
21. Stratonovich, R. L. *Topics in the Theory of Random Noise* Vol. I (Gordon & Breach, New York, 1981).
22. Lipsitz, L. A. *et al.* *Br. Heart J.* **74**, 340–396 (1995).
23. Gerstein, G. L. & Mandelbrot, B. B. *Blophys. J.* **4**, 41–68 (1964).

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## A mechanism for halogen release from sea-salt aerosol in the remote marine boundary layer

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RECENT measurements of inorganic chlorine gases<sup>1</sup> and hydrocarbons<sup>2</sup> indicate the presence of reactive chlorine in the remote marine boundary layer; reactions involving chlorine and bromine can affect the concentrations of ozone, hydrocarbons and cloud condensation nuclei. The known formation mechanisms of reactive halogens require significant concentrations of nitrogen oxides<sup>3–5</sup>, which are not present in the unpolluted air of the remote marine boundary layer<sup>6</sup>. Here we propose an autocatalytic mechanism for halogen release from sea-salt aerosol: gaseous HOBr is scavenged by the aerosol and converted to only slightly soluble BrCl and Br<sub>2</sub>, which are released into the gas phase. Depending on the sea-salt concentration and given a boundary layer that is stable for a few days, gaseous HOCl and HOBr may reach molar mixing ratios of up to 35 pmol mol<sup>-1</sup>. We calculate that HOBr and HOCl are responsible for 20% and 40%, respectively, of the sulphur (iv) oxidation<sup>7,8</sup> that occurs in the aerosol phase. The additional S(iv) oxidation reduces the formation of cloud-condensation nuclei, and hence the feedback between greenhouse warming, oceanic DMS emission and cloud albedo. We also calculate significant bromine-catalysed ozone loss.

Recently, considerable attention<sup>9–13</sup> has been given to the role in the chemistry of the marine boundary layer (MBL) of chlorine atoms, which react with alkanes up to two orders of magnitude faster than do hydroxyl radicals. Chlorine atoms may accordingly serve as an additional oxidant, at concentrations larger than 1 × 10<sup>3</sup> atoms cm<sup>-3</sup>. From diurnal measurements of non-methane hydrocarbons<sup>2,14</sup>, or the observation of inorganic chlorine gases, Cl-atom concentrations of the order of 10<sup>4</sup>–10<sup>5</sup> atoms cm<sup>-3</sup> were inferred. In addition, Barrie *et al.*<sup>15</sup> have suggested—and there is now evidence for this from field measurements<sup>16</sup>—that ozone is destroyed in the MBL during polar sunrise by a mechanism involving Br and BrO.

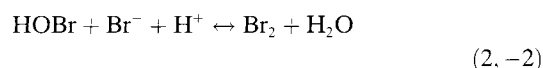
So far no satisfactory mechanism has been proposed for

reactive bromine and chlorine production in the pristine MBL or the Arctic. The gas-phase reactions of OH radicals with HCl or HBr are only a minor source of halogen atoms. Singh and Kasting<sup>17</sup> calculated Cl-atom concentrations of 10<sup>3</sup> atoms cm<sup>-3</sup>, assuming an HCl volume mixing ratio of 1 nmol mol<sup>-1</sup>, which is much larger than measured in the remote MBL<sup>1,2,18</sup>. Very recently, Mozurkewich<sup>19</sup> suggested the reaction of peroxomonosulphuric (Caro's) acid (HSO<sub>5</sub>) with sea-salt bromide as a source of elemental bromine in the Arctic. However, the mechanism is favoured by low temperatures and high SO<sub>2</sub> concentrations and by itself should not oxidize significant amounts of halides. Mozurkewich also discussed direct bromide oxidation through free radicals, such as OH or HO<sub>2</sub> in the sea-salt aerosol. However, none of these mechanisms is capable of significant chlorine atom production in the unpolluted MBL.

Our proposed mechanism for autocatalytic bromine and chlorine chemistry in the liquid and gas phase is shown in Fig. 1. Hypobromous acid, HOBr, which is formed through an initial bromide oxidation (see below), is scavenged by sea-salt aerosol. Because in sea water (and therefore also in the nascent sea-salt aerosol) the bromide to chloride ratio is ~1/700, HOBr reacts with Cl<sup>-</sup>:

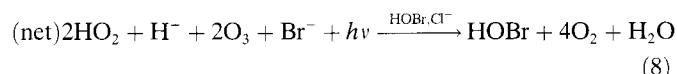
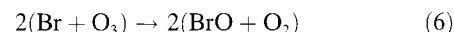
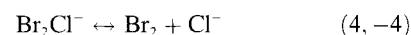
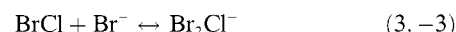


Recently, Wang *et al.*<sup>20</sup> determined a lower limit of the BrCl hydrolysis rate constant,  $k_{-1} \geq 1 \times 10^5 \text{ s}^{-1}$ . From the equilibrium constant  $K_{1\text{eq}} = 5.6 \times 10^4 \text{ M}^{-2}$  (ref. 20), we calculate the rate constant of the forward reaction  $k_1 \geq 5.6 \times 10^9 \text{ M}^{-2} \text{ s}^{-1}$ , which is of the same order of magnitude as that of the comparable reaction of HOBr with Br<sup>-</sup> (ref. 21).



$$(k_2 = 1.6 \times 10^{10} \text{ M}^{-2} \text{ s}^{-1}, k_{-2} = 110 \text{ s}^{-1})$$

This reaction has been discussed as potentially important for bromine cycling on sulphate aerosol<sup>22</sup> and for autocatalytic bromine release from sea-salt aerosol<sup>19,23</sup>. Because of the large [Cl<sup>-</sup>]/[Br<sup>-</sup>] ratio in sea water, the forward reaction (1) is much more important than forward reaction (2). Although the hydrolysis reaction (-1) is faster than reaction (-2), this is of lesser significance because a substantial fraction of the BrCl will react with Br<sup>-</sup> leading to autocatalytic Br activation:



Equilibria (3) and (4) were found to be established very rapidly<sup>20</sup>, probably at a diffusion-controlled rate. After escape of Br<sub>2</sub> to the gas phase, photolysis (5) and reactions (6), (7) and (1) an autocatalytic cycle of bromine activation is closed. Thus, bromide oxidation is driven by HO<sub>2</sub>, O<sub>3</sub> and aerosol acidity in the presence of sunlight and is catalysed by HOBr and Cl<sup>-</sup>.

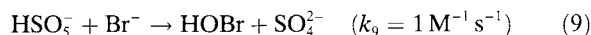
We have incorporated reactions (1)–(7) into a photochemical box model of the MBL<sup>23</sup>. The model treats chemical reactions in the gas phase and deliquesced sea-salt particles, as well as exchange between the two phases; standard O<sub>3</sub>–NO<sub>x</sub>–HO<sub>x</sub>–S

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chemistry<sup>23</sup> is used. The sea-salt aerosol concentration was  $3 \times 10^{-11} \text{ m}^3$  of liquid per  $\text{m}^3$  of air (that is,  $9.4 \mu\text{g NaCl}$  per  $\text{m}^3$  of air) with a size distribution from ref. 24, yielding a volume geometric median particle radius of  $3 \mu\text{m}$ , a number density of  $0.7 \text{ cm}^{-3}$  and a surface area of  $30 (\mu\text{m})^2 \text{ cm}^{-3}$ , assuming that sea salt represents the coarse fraction of the aerosol. The liquid-phase concentrations are averaged over the entire particle ensemble which is assigned a lifetime of 2 days. Fresh and slightly alkaline aerosol is added continuously while all liquid-phase compounds are removed with the lifetime of the aerosol. We recognize that our treatment of the sea salt is a major simplification, which neglects effects of lower pH that is established in smaller particles.

For some runs we also included a similar scheme of reactions on the sub-micrometre mode of marine aerosol. Typical background aerosol concentration,  $1.1 \times 10^{-12} \text{ m}^3$  (liquid) per  $\text{m}^3$  (air), size distribution (volume geometric median radius of  $0.1 \mu\text{m}$ ), and chemical composition (mainly  $\text{NH}_4\text{HSO}_4$ ,  $(\text{NH}_4)_2\text{SO}_4$  and water) are taken from MBL measurements<sup>25</sup>. Scavenging of HOBr, HCl and HBr on the additional surface of the submicrometre sulphate aerosol in combination with reactions (1) and (2) also leads to efficient conversion of  $\text{Cl}^-$  and  $\text{Br}^-$  into BrCl and  $\text{Br}_2$ .

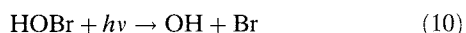
Figure 2a–c show the gas-phase halogen,  $\text{HO}_x$  and  $\text{NO}_x$  concentrations for the first two days of a model run (no sulphate aerosol) which was started without gas-phase halogens present. Owing to rapid uptake of  $\text{SO}_2$  from the gas phase and subsequent oxidation to  $\text{SO}_4^{2-}$ , the acidity of the originally alkaline sea-salt aerosol particles drops to about pH 5.5. This causes some HCl to evaporate, yielding a gas-phase concentration of HCl of approximately  $20 \text{ pmol mol}^{-1}$ . During the first 6 hours of the model run (night) the only radical species present is  $\text{NO}_3$ . A fraction of the  $\text{NO}_3$  is scavenged by the sea-salt aerosol, where a free-radical chain oxidation of S(IV) (refs 19, 26) is started, followed by Caro's acid ( $\text{HSO}_5^-$ ) production, oxidation of  $\text{Br}^-$  to HOBr



and  $\text{Br}_2$  formation via reactions (1), (3) and (4). At dawn,  $\text{Br}_2$  at a concentration of  $0.06 \text{ pmol mol}^{-1}$  has formed and after its rapid photolysis the autocatalytic bromide oxidation described above takes over. After the second day of cycling, BrCl and  $\text{Br}_2$  reach early-morning maxima of 3.8 and  $2.2 \text{ pmol mol}^{-1}$ , respectively, and a daytime HOBr concentration of  $7 \text{ pmol mol}^{-1}$  is calculated. Bromine in particular is very efficiently transferred to the gas phase, causing a bromide deficit of about 90%. The calculated chloride deficit is about 1% after two days of cycling.

Steady-state concentrations are reached after  $\sim 14$  model days. The daytime maxima ( $\text{pmol mol}^{-1}$ ) are:  $[\text{HCl}] = 28$ ,  $[\text{HOCl}] = 2.7$ ,  $[\text{HOBr}] = 13$  and  $[\text{HBr}] = 1.9$ . Night-time maxima ( $\text{pmol mol}^{-1}$ ) are  $[\text{BrCl}] = 7.5$ ,  $[\text{Br}_2] = 3$ ,  $[\text{ClONO}_2] = 1.3$ ,  $[\text{BrONO}_2] = 1.2$  and  $[\text{Cl}_2] = 0.4$ . Although it may be unrealistic to assume that an air mass will stay in the MBL for such a long period, this assumption gives some insight in how rapidly the autocatalytic cycle might work. After only two days of cycling,  $\sim 50\%$  of the steady-state halogen concentrations are reached.

The bromine atoms formed upon photolysis of  $\text{Br}_2$  or BrCl react mainly with ozone. Significant amounts of ozone can be destroyed in catalytic cycles, involving reactions (6) and (7), and HOBr photolysis:



The calculated amount of ozone destroyed during the second day of sea-salt processing is  $\sim 0.14 \text{ nmol mol}^{-1}$ . This compares to about 2.5 and  $1.2 \text{ nmol mol}^{-1} \text{ d}^{-1}$  of ozone destruction through photo-

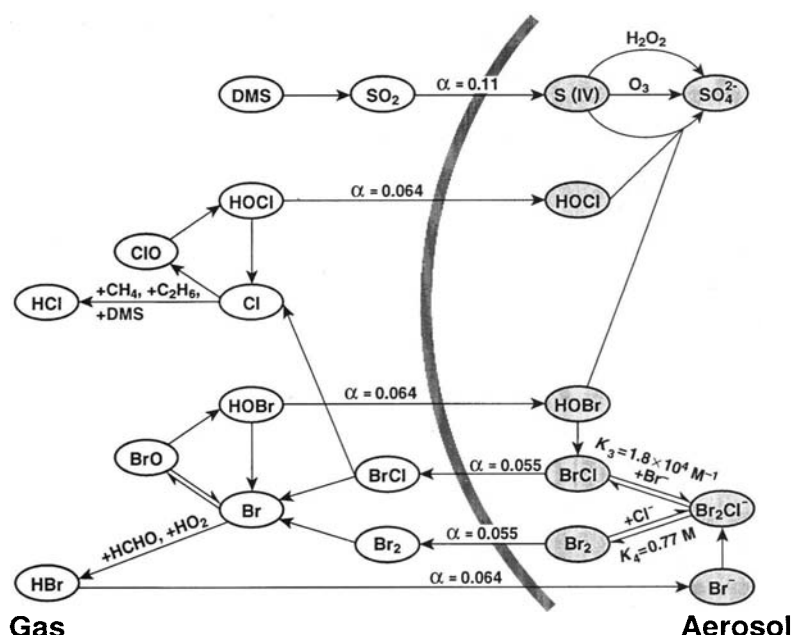


FIG. 1 A simplified scheme of halogen cycling in the MBL. The transformations of compounds in the shaded ovals occur in the deliquesced sea-salt aerosol. Also given are the accommodation coefficients,  $\alpha$ , and equilibrium constants,  $K$ , used in the model calculations.

lysis ( $\text{O}_3 \rightarrow \text{O}(^1\text{D})$ ;  $\text{O}(^1\text{D}) + \text{H}_2\text{O} \rightarrow 2\text{OH}$ ) and chemical reactions ( $\text{OH} + \text{O}_3 \rightarrow \text{HO}_2 + \text{O}_2$ ,  $\text{HO}_2 + \text{O}_3 \rightarrow \text{OH} + 2\text{O}_2$ ) at  $\text{O}_3$  concentrations of 40 or  $20 \text{ nmol mol}^{-1}$ , respectively. Although the Br-catalysed ozone destruction during the second model day is only 5–10% of the total ozone loss, we calculate at steady state a destruction of 20–40%, if recycling of HBr and HCl on sulphate aerosol is also considered.

A crucial parameter is the sea-salt aerosol content, which is largely determined by the wind speed. If we assume a 5 times higher aerosol content ( $15 \times 10^{-11} \text{ m}^3$  (liquid), per  $\text{m}^3$  (air), that is,  $47 \mu\text{g NaCl}$  per  $\text{m}^3$  (air)), which is at the upper range of values typically found in the MBL, the reactive halogen concentrations increase dramatically. We estimate maximum concentrations ( $\text{pmol mol}^{-1}$ ) after two days of  $[\text{HCl}] = 70$ ,  $[\text{HOCl}] = 33$ ,  $[\text{HOBr}] = 35$ ,  $[\text{HBr}] = 3$ ,  $[\text{BrCl}] = 38$ ,  $[\text{Br}_2] = 10$  and  $[\text{Cl}_2] = 1.5$ . Catalytic ozone loss through bromine and chlorine during the second day increases to 1.4 or  $0.6 \text{ nmol mol}^{-1}$ , respectively, corresponding to 58% or 25% of the ozone lost by photolysis and  $\text{HO}_x$  chemistry.

The proposed autocatalytic mechanism also represents a source of chlorine atoms via the photolysis of BrCl. The product of reaction (1), BrCl, like  $\text{Br}_2$  or  $\text{Cl}_2$  is only slightly soluble. In the gas phase BrCl will undergo fast photolysis:



Chlorine-atom concentrations reach  $1 \times 10^3 \text{ atoms cm}^{-3}$  during the second day of simulation, and  $2.5 \times 10^3 \text{ atoms cm}^{-3}$  at steady state. Including recycling reactions on sulphate aerosol particles has a strong effect: Cl-atom concentrations are increased by a factor of 3.5 and reach  $10^4 \text{ atoms cm}^{-3}$  at steady state. With a five times higher sea-salt aerosol content, we estimate for the second day of processing  $[\text{Cl}] = 3 \times 10^4 \text{ atoms cm}^{-3}$ , and  $[\text{Cl}] = 5 \times 10^4 \text{ atoms cm}^{-3}$ , if we also include sulphate aerosol particles. The strong nonlinear response of the Cl concentration to the sea-salt aerosol content is caused by the multiplication effect of higher HOBr concentration and the reaction chain (1), (11), (6–7).

Our mechanism of Br activation can thus also account for the presence of Cl atoms in MBL<sup>1,2</sup>. The simulated overnight

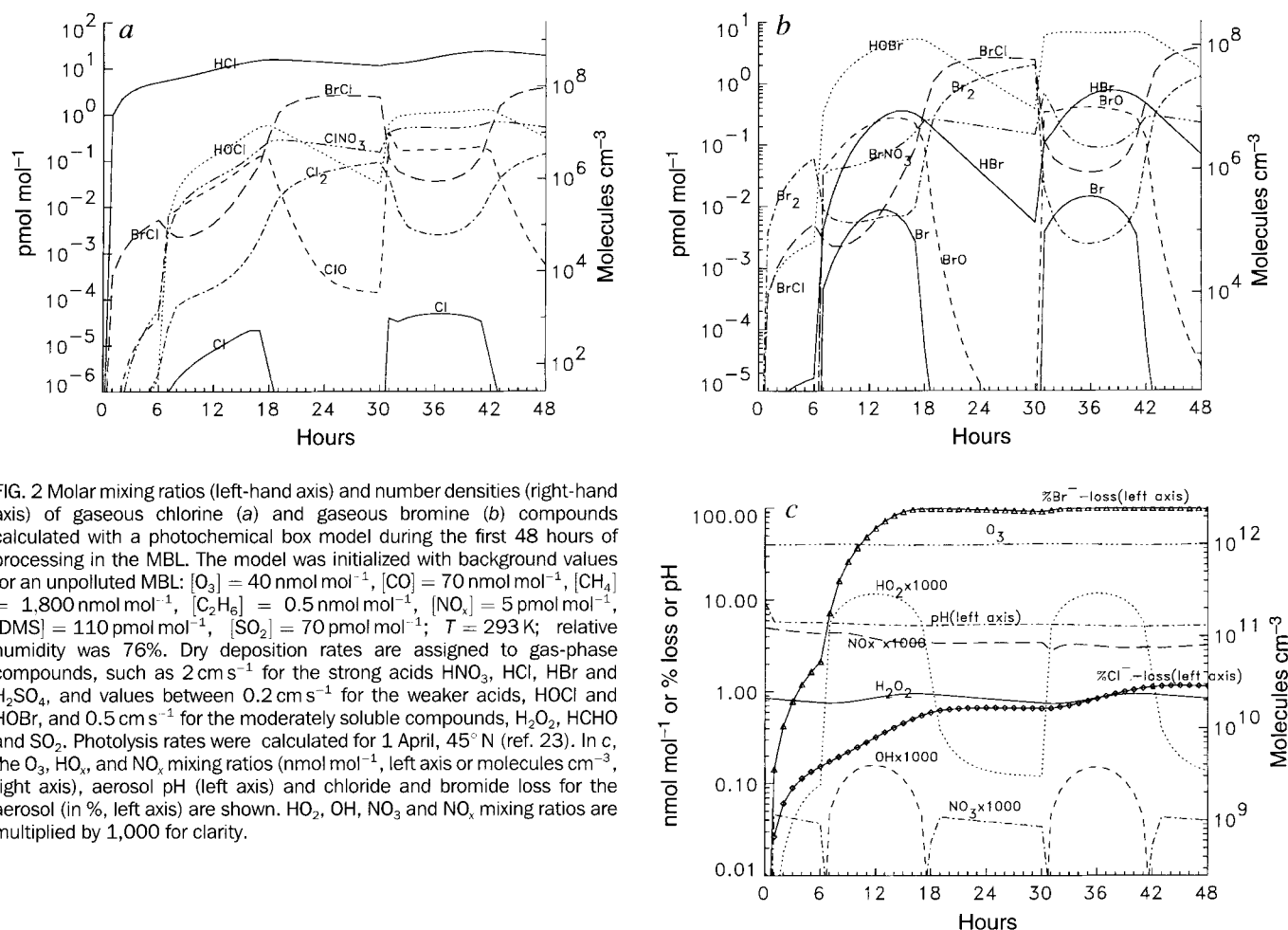
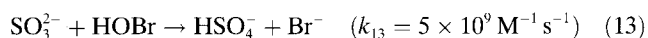


FIG. 2 Molar mixing ratios (left-hand axis) and number densities (right-hand axis) of gaseous chlorine (a) and gaseous bromine (b) compounds calculated with a photochemical box model during the first 48 hours of processing in the MBL. The model was initialized with background values for an unpolluted MBL:  $[\text{O}_3] = 40 \text{ nmol mol}^{-1}$ ,  $[\text{CO}] = 70 \text{ nmol mol}^{-1}$ ,  $[\text{CH}_4] = 1,800 \text{ nmol mol}^{-1}$ ,  $[\text{C}_2\text{H}_6] = 0.5 \text{ nmol mol}^{-1}$ ,  $[\text{NO}_x] = 5 \text{ pmol mol}^{-1}$ ,  $[\text{DMS}] = 110 \text{ pmol mol}^{-1}$ ,  $[\text{SO}_2] = 70 \text{ pmol mol}^{-1}$ ;  $T = 293 \text{ K}$ ; relative humidity was 76%. Dry deposition rates are assigned to gas-phase compounds, such as  $2 \text{ cm s}^{-1}$  for the strong acids  $\text{HNO}_3$ ,  $\text{HCl}$ ,  $\text{HBr}$  and  $\text{H}_2\text{SO}_4$ , and values between  $0.2 \text{ cm s}^{-1}$  for the weaker acids,  $\text{HOCl}$  and  $\text{HOBr}$ , and  $0.5 \text{ cm s}^{-1}$  for the moderately soluble compounds,  $\text{H}_2\text{O}_2$ ,  $\text{HCHO}$  and  $\text{SO}_2$ . Photolysis rates were calculated for 1 April,  $45^\circ \text{ N}$  (ref. 23). In c, the  $\text{O}_3$ ,  $\text{HO}_x$  and  $\text{NO}_x$  mixing ratios ( $\text{nmol mol}^{-1}$ , left axis or  $\text{molecules cm}^{-3}$ , right axis), aerosol pH (left axis) and chloride and bromide loss for the aerosol (in %, left axis) are shown.  $\text{HO}_2$ ,  $\text{OH}$ ,  $\text{NO}_3$  and  $\text{NO}_x$  mixing ratios are multiplied by 1,000 for clarity.

accumulation of a Cl- and Br-atom precursor in conjunction with the daytime formation of HCl agrees with diel patterns observed by Pszenny *et al.*<sup>1</sup>. The main fate of Cl atoms is reaction with ozone, and to a smaller extent with methane. The additional methane loss on the second model day is small ( $\sim 2\%$ , considering recycling on sulphate aerosol), but may reach 18%, if the sea-salt aerosol content is increased by a factor of five. If recycling on sulphate aerosol is also taken into account, the additional methane loss is 26%. For ethane, we calculate an additional loss of the order of 10%, or 30% considering recycling on sulphate particles. If the sea-salt aerosol content is increased by a factor of five, ethane loss is dominated by Cl and the total loss is 3.9 times larger than by OH reaction alone.

HOBr and HOCl are scavenged by the aerosol (Fig. 1). There they are capable of very rapid S(IV) oxidation<sup>7,8</sup>:



The rate coefficients of reactions (12) and (13) have been measured at high pH values of 10–9. The rate constants were found to increase at lower pH. If we adopt for the analogous reactions of  $\text{HSO}_3^-$  (which is the predominant S(IV) species at pH range 3–6) the same rate constants as for  $\text{SO}_3^{2-}$ , we calculate on the second model day that  $\sim 40\%$  of the  $\text{SO}_2$  scavenged by the aerosol is oxidized by HOCl and  $\sim 20\%$  by HOBr. The remaining oxidation occurs by ozone,  $\text{H}_2\text{O}_2$  (ref. 26) and free radicals, such as  $\text{Cl}_2^-$  and  $\text{Br}_2^-$ . But the total amount of S(IV) oxidized in sea-salt aerosol does not only depend on the availability of oxidants, but also on the transport of  $\text{SO}_2$  into the aerosol particle and the solubility of  $\text{SO}_2$ . We estimate that the total amount of S(IV)

oxidized in sea-salt aerosol is increased by 0.4–0.8. If we include recycling reactions on sulphate aerosol, these values increase to 2.4–3.6. The additional  $\text{SO}_2$  oxidation on pre-existing particles reduces the formation of new cloud-condensation nuclei and therefore the feedback between greenhouse warming, oceanic dimethyl sulphide emissions and increased cloud albedo<sup>27,28</sup>.

The proposed autocatalytic mechanism critically depends on the rate of reaction (1). Our calculations stress the need for improved kinetic data of hypohalogenic acids in concentrated halogenide solutions. Because the  $\text{H}^+$  concentration determines the rate of reaction (1), better knowledge of aerosol pH is also required. Furthermore the Henry's law constant ( $H$ ) of HOBr needs to be determined. We have assumed  $H_{\text{HOBr}} = 92.6 \text{ M atm}^{-1}$ , which is 1/10 of the solubility constant of HOCl at 293 K (ref. 29). In sensitivity studies, we find that the value of  $H_{\text{HOBr}}$  can be lowered to  $\sim 0.1 \text{ M atm}^{-1}$  until HOBr solubility limits the mechanism suggested.

The calculated reactive halogen concentrations of  $10\text{--}40 \text{ pmol mol}^{-1}$  should be detectable in the field. The main daytime species are HOBr and HOCl, whereas at night BrCl and  $\text{Br}_2$  are most abundant. Our calculated daytime BrO concentrations reach values of  $\sim 10^7\text{--}10^8 \text{ molecules cm}^{-3}$ , which is in the detection range of differential optical absorption spectroscopy (DOAS). Some experimental evidence already exists<sup>30</sup> for the proposed  $\text{Br}^-$ -catalysed production of Cl from sea-salt aerosol, which was irradiated in a large reaction chamber in the presence of  $\text{O}_3$ . □

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1. Pszenny, A. A. P. *et al.* *Geophys. Res. Lett.* **20**, 699–702 (1993).
2. Singh, H. B. *et al.* *J. Geophys. Res.* **101**, 1907–1917 (1996).
3. Finlayson-Pitts, B. J. *Res. Chem. Intermediates* **19**, 235–249 (1993).



4. Zetzsch, C. & Behnke, W. *Ber. Bunsenges. Phys. Chem.* **96**, 488–493 (1992).
5. Finlayson-Pitts, B. J., Livingston, F. E. & Berk, H. N. *J. Phys. Chem.* **93**, 4397–4400 (1989).
6. Rohrer, F. & Brüning, D. *J. Atmos. Chem.* **15**, 253–267 (1992).
7. Fogelman, K. D., Walker, D. M. & Margerum, D. W. *Inorg. Chem.* **28**, 986–993 (1989).
8. Troy, R. C. & Margerum, D. W. *Inorg. Chem.* **30**, 3538–3543 (1991).
9. Finlayson-Pitts, B. J. *J. Geophys. Res.* **98D**, 14991–14993 (1993).
10. Parrish, D. D. *et al. J. Geophys. Res.* **98D**, 14995–14997 (1993).
11. McKeen, S. A. & Liu, S. C. *Geophys. Res. Lett.* **20**, 2363–2366 (1993).
12. Wingenter, O. W. *et al. J. Geophys. Res.* **101**, 4331–4340 (1996).
13. Graedel, T. E. & Keene, W. C. *Global Biogeochem. Cycles* **9**(1), 47–77 (1995).
14. Jobson, B. T. *et al. J. Geophys. Res.* **99D**, 25355–25368 (1994).
15. Barrie, L. A., Bottenheim, J. W., Schnell, R. C., Crutzen, P. J. & Rasmussen, R. A. *Nature* **334**, 138–141 (1988).
16. Hausmann, M. & Platt, U. *J. Geophys. Res.* **99D**, 25399–25413 (1994).
17. Singh, H. B. & Kasting, J. F. *J. Atmos. Chem.* **7**, 261–285 (1988).
18. Harris, G. W., Klemp, D. & Zenker, T. *J. Atmos. Chem.* **15**, 327–332 (1992).
19. Mozurkewich, M. J. *Geophys. Res.* **100D**, 14199–14207 (1995).
20. Wang, T. X., Kelley, M. D., Cooper, J. N., Beckwith, R. C. & Margerum, D. W. *Inorg. Chem.* **33**, 5872–5878 (1994).
21. Eigen, M. & Kustin, K. *J. Am. Chem. Soc.* **84**, 1355–1361 (1962).
22. Fan, S.-M. & Jacob, D. J. *Nature* **359**, 522–524 (1992).
23. Sander, R. & Crutzen, P. J. *J. Geophys. Res.* **101**, 9121–9138 (1996).
24. Kim, Y., Sievering, H. & Boatman, J. *Glob. Biogeochem. Cycles* **4**, 165–177 (1990).
25. Kim, Y., Sievering, H., Boatman, J., Wellman, D. & Pszenny, A. J. *Geophys. Res.* **100**, 23027–23038 (1995).
26. Chameides, W. L. & Stelson, A. W. J. *Geophys. Res.* **97D**, 20565–20580 (1992).
27. Charlson, R. J., Lovelock, J. E., Andreae, M. O. & Warren, S. G. *Nature* **326**, 655–661 (1987).
28. Sievering, H. *et al. Nature* **360**, 571–573 (1992).
29. Huthwelker, T. *et al. J. Atmos. Chem.* **21**, 81–95 (1995).
30. Behnke, W., Elend, M., Krüger, H. U., Scheer, V. & Zetzsch, C. in *Proc. Eurotrac Symp.* (eds Borrell, P. M. *et al.*) (Computational Mechanics, Southampton, in the press).

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## The role of heterotrophic bacteria in iron-limited ocean ecosystems

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**IRON availability limits phytoplankton growth in large areas of the world's oceans<sup>1–3</sup> and may influence the strength of the biological carbon pump<sup>4,5</sup>. Very little is known of the iron requirements of oceanic heterotrophic bacteria, which constitute up to 50% of the total particulate organic carbon in open ocean waters<sup>6,7</sup> and are important in carbon cycling as remineralizers of dissolved organic matter and hence producers of CO<sub>2</sub> (ref. 8). Here we report that oceanic bacteria contain more iron per biomass than phytoplankton. In the subarctic Pacific, they constitute a large fraction of biogenic iron and account for 20–45% of biological iron uptake. Bacterial iron quotas in the field are similar to those of iron-deficient laboratory cultures, which exhibit reduced electron transport, slow growth, and low carbon growth efficiency. Heterotrophic bacteria therefore play a major role in the biogeochemical cycling of iron. *In situ* iron limitation of heterotrophic metabolism may have profound effects on carbon flux in the ocean.**

We measured intracellular iron concentrations of five oceanic bacterial isolates maintained in synthetic ocean water medium<sup>9</sup> enriched with an organic carbon substrate extracted from a diatom culture. Under iron-sufficient conditions (concentrations of inorganic iron, [Fe<sup>2+</sup>] = 40 nM), bacterial iron quotas averaged 44.0 μmol Fe:mol C, and decreased to a mean value of 7.5 in iron-deficient culture medium ([Fe<sup>2+</sup>] = 25 pM). These latter Fe quotas were low enough to inhibit growth of a number of isolates and can therefore be taken as an estimate of the minimal iron requirement of open-ocean, heterotrophic bacteria. Comparison of these Fe:C ratios with those of oceanic phytoplankton<sup>10</sup> cultured under the same iron-limiting conditions indicates that the heterotrophic bacteria have a higher iron content than eukaryotic phototrophs (Table 1).

To substantiate these laboratory results, we measured Fe:C ratios of natural assemblages of bacterioplankton and phytoplankton at ocean station P (OSP: 50° N, 145° W) in the subarctic Pacific Ocean during September 1995. This ocean region is iron-deficient<sup>3</sup> and has high concentrations of major nutrients and low chlorophyll (HNLC). The iron quotas of both heterotrophic bacteria and phytoplankton in these waters were not significantly different from those measured in iron-limited cultures (Table 1). The Fe:C

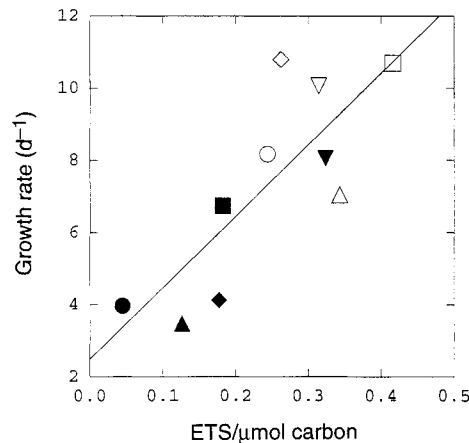


FIG. 1 Relationship between growth rate and electron transport system activity (ETS) in two oceanic, Jul 88 (○) and Tef 2 (□), and three coastal bacterial isolates, Lmb1 (Δ), Lmg1 (▽) and Pwh3a *Vibrio natrigens* (◇), under iron-sufficient (open symbols) ([Fe<sup>2+</sup>] = 40 nM) and iron-deficient (filled symbols) conditions ([Fe<sup>2+</sup>] = 25 pM). The plotted line was obtained by least-squares regression ( $r^2 = 0.65$ ;  $P = 0.005$ ).

ratios of heterotrophic bacteria were twofold higher than those of phytoplankton in these iron-poor waters (Table 1).

The bacterial Fe:C ratios reported here bring new insight into the biological partitioning of iron in the ocean. Table 1 shows a biogenic iron budget for OSP, based on our Fe:C measurements combined with published estimates of heterotrophic bacteria and phytoplankton biomass in these waters. Cyanobacteria have been included in the analysis, using the Fe:C ratio reported from a laboratory study of a single Pacific Ocean strain<sup>11</sup>, although field measurements are lacking. Total biogenic iron determined in this way is ~20 pM, substantially lower than the 400 pM previously reported<sup>12</sup>. The reason for this discrepancy is unclear, although our estimate may be low because we have neglected a small (albeit significant) amount of iron contained in heterotrophic protozoans (Z. Chase and N.M.P., manuscript submitted) and mesozooplankton<sup>13</sup>.

The most striking result of our calculation is that heterotrophic bacteria constitute the largest fraction of biogenic iron in this system (~50% of the total) (Table 1). Bacteria also seem to assimilate a substantial amount of the available iron in these waters. Steady-state iron-uptake rates, calculated from the total iron in each biological pool and the mean turnover rates, show that heterotrophic bacteria account for ~20% of total community uptake at OSP (Table 1). Our calculation may overestimate cyanobacterial iron-uptake rates (and hence underestimate the importance of heterotrophic uptake) if their Fe:C ratio measured in the laboratory is substantially higher than *in situ*. In addition, the relative contribution of each biological compartment to community iron uptake will be influenced by spatial and temporal variability<sup>14</sup> in turnover times.

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TABLE 1 Iron content and assimilation rates of autotrophic and heterotrophic plankton

| Plankton group           | Laboratory Fe:C<br>( $\mu\text{mol}:\text{mol}$ ) | Field Fe:C<br>( $\mu\text{mol}:\text{mol}$ ) | Biomass ( $\mu\text{mol C l}^{-1}$ ) | Biogenic Fe<br>( $\text{pmol l}^{-1}$ ) | Turnover rate<br>( $\text{d}^{-1}$ ) | Fe uptake rate<br>( $\text{fmol Fe l}^{-1} \text{ h}^{-1}$ ) |
|--------------------------|---------------------------------------------------|----------------------------------------------|--------------------------------------|-----------------------------------------|--------------------------------------|--------------------------------------------------------------|
| Heterotrophic bacteria   | $7.52 \pm 1.65$                                   | $9.1 \pm 1.50$                               | $1.18 \pm 0.37$ (ref. 7)             | $10.7 \pm 3.8$                          | 0.06 (ref. 7)                        | 27                                                           |
| Cyanobacteria            | 19 (ref. 19)                                      |                                              | $0.24 \pm 0.16$ (ref. 26)            | 4.56                                    | 0.2 (ref. 27)                        | 38                                                           |
| Eukaryotic phytoplankton | $3.0 \pm 0.73$ (ref. 10)                          | $4.4 \pm 0.82$                               | $1.41 \pm 0.49$ (ref. 26)            | $6.2 \pm 0.96$                          | 0.25 (ref. 28)                       | 65                                                           |
| Total                    |                                                   |                                              | 2.83                                 | 21.5                                    |                                      | 130                                                          |

Laboratory Fe:C ratio ( $\pm$ s.d.) is the mean of 5 Sargasso Sea bacterial isolates grown in Fe-deficient (inorganic iron,  $[\text{Fe}'] = 25 \text{ pM}$ ) laboratory cultures. Fe:C ratios of field plankton were measured in size-fractionated samples from the surface-mixed layer of ocean station P, subarctic Pacific Ocean. Biomass and Fe:C ratios of plankton from OSP were used to determine biogenic iron, which was multiplied by turnover rate to estimate steady-state iron-uptake rates.

Size-fractionated  $^{55}\text{Fe}$ -uptake experiments show that bacteria (0.2–1.0  $\mu\text{m}$  size class) take up 16% of the dissolved iron at OSP, in agreement with the steady-state calculation. It was technically necessary to make these field measurements at higher than ambient iron levels (50 nM Fe complexed with 50  $\mu\text{M}$  EDTA was added to incubation bottles) and thus the measured total community uptake rate (2,800 fmol Fe per l per h) is greater than the calculated *in situ* value. Nonetheless, these experiments provide reasonable estimates of relative iron-uptake rates among size classes, although the addition of exogenous iron to the samples may favour uptake by larger cells, and thus underestimate relative uptake by bacteria. The contribution of bacteria to community iron uptake at OSP may have been further underestimated in our experiments as phytoplankton biomass (chlorophyll *a*,  $0.44 \mu\text{g l}^{-1}$ ) at the time of sampling was significantly higher than the ten-year average<sup>15</sup>. Indeed, at another iron-limited, subarctic Pacific site<sup>16</sup> (P4;  $48^\circ \text{N}$ ,  $126^\circ \text{W}$ ) with lower phytoplankton biomass (chlorophyll *a*,  $0.27 \mu\text{g l}^{-1}$ ), bacteria accounted for 47% of iron uptake. Experiments using the metabolic inhibitor streptomycin show that heterotrophic and phototrophic bacteria account for 30% of total community uptake. Overall, heterotrophic bacteria seem to account for 20–45% of iron uptake in the subarctic Pacific.

The quota and flux observations imply that heterotrophic bacteria compete directly with phytoplankton for iron, a limiting resource. Such competition may be mediated through the production and release of high-affinity iron-uptake ligands (siderophores) by heterotrophic bacteria<sup>17</sup>. Although speciation of iron in the Pacific is controlled by complexation reactions with organic ligands, whose binding constants resemble those of well-characterized siderophores<sup>18</sup>, the biological source(s) of these complexing agents remains unknown.

Because iron quotas of heterotrophic bacteria in the subarctic Pacific are similar to those in iron-deficient laboratory cultures (Table 1), these organisms may be iron limited *in situ*. We have characterized the physiological effects of iron limitation on a number of oceanic and coastal bacterial isolates maintained in laboratory batch cultures. The growth of 7 out of 11 isolates was significantly inhibited at inorganic iron concentrations (25 pM) higher than those in central north Pacific Ocean<sup>18</sup>, which permit maximum growth rates of many oceanic phytoplankton<sup>19,20</sup>. Reduction in growth rate was highly correlated to the relative decrease in cellular Fe:C in iron-deficient culture medium ( $r^2 = 0.67$ ;  $P < 0.05$ ). This correlation can be explained by an inhibition of respiratory electron transport system (ETS) activity (normalized to biomass) at low cellular Fe:C ratios, consistent with the high iron content of the respiratory chain<sup>21</sup>. Lower ETS activity was not simply a result of slower growth rates as the activity of  $\beta$ -glucosidase, a non-iron-dependent enzyme, was unaffected by cellular iron (isolate Jul 88: iron-sufficient enzyme activity, 0.16; iron-deficient culture, 0.17 relative fluorescence units per C per min). Among five bacterial strains examined, cellular ETS activities were highly correlated

to growth rates (Fig. 1), reflecting the dependence of biosynthetic rates on the production of ATP. Measurement of biomass-normalized ETS activity may therefore be a sensitive physiological biomarker of heterotrophic iron deficiency in the field.

The inhibition of ETS at low concentrations of iron significantly affected cellular metabolism without altering bulk biochemical composition (C:N ratio) (Table 2). Three of five iron-limited bacterial isolates showed lower carbon growth efficiency, respiring more organic substrate as  $\text{CO}_2$  and incorporating less into biomass (Table 2). Thus iron limitation in marine heterotrophic bacteria induces inefficient carbon metabolism, as previously reported in a terrestrial isolate<sup>22</sup>.

Iron-induced reduction in carbon growth efficiency could be particularly important in oceanic bacteria, given their role in global carbon cycling. Bacterial production is thought to be limited by the amount of dissolved organic substrate<sup>23</sup>. Iron deficiency would act to regulate the amount of this substrate that is incorporated into biomass compared with that which is respired as  $\text{CO}_2$ . Iron availability may therefore partly determine the relative contribution of the microbial loop to remineralization and carbon transfer to higher trophic levels. Our results demonstrate that marine heterotrophic bacteria play a much larger role in the biogeochemical cycling of iron than previously recognized, and highlight the need for a more complete understanding of how iron may regulate biological productivity and carbon flux in the ocean. □

## Methods

**Determination of Fe:C ratios.** Fe:C ratios of 5 bacterial isolates from the Sargasso Sea (including *Pseudomonas* spp.) were measured in iron-sufficient (inorganic iron,  $[\text{Fe}'] = 40 \text{ nM}$ ) and iron-deficient ( $[\text{Fe}'] = 25 \text{ pM}$ ), laboratory cultures maintained in artificial sea water<sup>9</sup> containing  $^{55}\text{Fe}$  ( $0.44 \text{ Ci mol}^{-1}$ ; Du Pont) and  $^{14}\text{C}$ -labelled organic substrate. The  $^{14}\text{C}$  substrate was extracted from 40 l of  $^{14}\text{C}$ - $\text{HCO}_3^-$  labelled ( $5 \mu\text{Ci l}^{-1}$ ;  $8.4 \text{ Ci mol}^{-1}$ ; Du Pont) *Thalassiosira weissflogii*, and trace metal contaminants removed using Chelex-100 resin<sup>9</sup>. Bacteria were allowed to complete 8 cell divisions to ensure intracellular isotopic equilibrium, and were collected by filtration and rinsed with  $\text{Ti(III)}$  citrate EDTA reagent to remove extracellular iron<sup>24</sup>. Fe:C ratios of field plankton were

TABLE 2 Carbon growth efficiency and C:N ratios of bacterial isolates under iron-sufficient and iron-deficient conditions

| Isolate | Carbon growth efficiency                          |                                                  | C:N ratio                                         |                                                  |
|---------|---------------------------------------------------|--------------------------------------------------|---------------------------------------------------|--------------------------------------------------|
|         | Iron-sufficient<br>$[\text{Fe}'] = 40 \text{ nM}$ | Iron-deficient<br>$[\text{Fe}'] = 25 \text{ pM}$ | Iron-sufficient<br>$[\text{Fe}'] = 40 \text{ nM}$ | Iron-deficient<br>$[\text{Fe}'] = 25 \text{ pM}$ |
| Jul 88* | $0.38 \pm 0.012$                                  | $0.12 \pm 0.020$                                 | $3.39 \pm 0.65$                                   | $3.87 \pm 0.34$                                  |
| Isol. 5 | $0.39 \pm 0.034$                                  | $0.36 \pm 0.022$                                 | $4.37 \pm 0.43$                                   | $3.68 \pm 0.9$                                   |
| Lmb1*   | $0.38 \pm 0.035$                                  | $0.23 \pm 0.012$                                 | $4.81 \pm 1.30$                                   | $4.36 \pm 0.18$                                  |
| Lmg1*   | $0.35 \pm 0.031$                                  | $0.23 \pm 0.029$                                 | $3.76 \pm 0.17$                                   | $3.73 \pm 0.43$                                  |
| Pwh3a   | $0.31 \pm 0.022$                                  | $0.36 \pm 0.026$                                 | $3.56 \pm 0.31$                                   | $4.29 \pm 0.63$                                  |

The first two bacterial strains were isolated from the Sargasso Sea and the last three from coastal waters of the Gulf of Mexico.

\* These isolates showed significantly lower growth efficiency in iron-deficient cultures ( $P < 0.05$ ). C:N ratios were unaffected by iron levels in all isolates.

measured in samples from the surface mixed layer of ocean station P (50° N, 145° W), subarctic Pacific Ocean. Water (20 l) from 10 m depth was collected using trace-metal clean techniques<sup>4</sup> and incubated on deck at *in situ* temperature and ambient light for 6 days with additions of  $\text{H}^{14}\text{CO}_3^-$  (2.4  $\mu\text{M}$ , 8.4 Ci mol<sup>-1</sup>) and  $^{55}\text{Fe}$  (5 nM, 0.44 Ci mol<sup>-1</sup> complexed to 100  $\mu\text{M}$  EDTA). Samples were collected on filters of varying porosity (0.2, 0.4, 1.0, 3.0, 5.0, 18.0  $\mu\text{m}$ ) and rinsed with Ti reagent<sup>24</sup>. Chlorophyll *a* concentration did not increase significantly during the incubation, indicating that growth of the natural community was not stimulated by the small  $^{55}\text{Fe}$  addition. Bacterial Fe:C ratios were obtained from the 0.2–0.4- $\mu\text{m}$  and 0.4–1.0- $\mu\text{m}$  size classes, because heterotrophic bacteria accounted for 98% of total cells counted in these fractions. Carbon quotas of bacteria obtained from  $^{14}\text{C}$  were 2.6 fmol per cell, similar to those reported previously<sup>25</sup>. The Fe:C ratio of phytoplankton was taken from the size classes >5  $\mu\text{m}$ .

**Iron-uptake experiments.** Sea water collected from 10 m depth at OSP was incubated on deck at *in situ* temperature and ambient light for 24 h with addition of 50 nM  $^{55}\text{Fe}$  complexed to 50  $\mu\text{M}$  EDTA. Every 4 h, samples were collected on filters of varying porosity and rinsed with Ti reagent<sup>24</sup>. Uptake rates were calculated by linear regression of particulate Fe per ml against time. Size

classes for bacteria and phytoplankton were those used for iron quota experiments. For inhibitor experiments, 30  $\mu\text{g}$  streptomycin per ml was used, and all samples were collected on 0.2- $\mu\text{m}$  filters.

**Growth rates and electron transport system (ETS) activity.** Bacterial isolates were maintained in medium enriched with 0.5 g l<sup>-1</sup> Casamino acids and Bacto-Peptone as carbon sources. Growth rates were determined in cultures inoculated into sterile, acid-washed polystyrene cuvettes which were incubated at 20 °C in a temperature-controlled spectrophotometer. Absorbance (510 nm) was measured every 10 min, and exponential growth rates were calculated from logarithmically transformed data by linear regression. ETS activity was measured in exponential-phase batch cultures<sup>29</sup>.

**Determination of carbon growth efficiency.** Bacteria grown with  $^{14}\text{C}$ -labelled organic substrate were collected in exponential phase by filtration onto 0.2- $\mu\text{m}$  filters. Carbon growth efficiency was calculated as the ratio of particulate organic carbon produced ( $^{14}\text{C}$  > 0.2  $\mu\text{m}$ ) to dissolved organic carbon consumed (<0.2  $\mu\text{m}$ ; initial acid-stable  $^{14}\text{C}$  – final acid-stable  $^{14}\text{C}$ ). C:N ratios were measured with a Carlo Erba EA-1108 elemental analyser after collecting exponential phase cells on GF/F filters (mean cell recovery ~95%).

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- Martin, J. H. et al. *Nature* **371**, 123–129 (1994).
- de Baar, H. J. W. et al. *Nature* **373**, 412–415 (1995).
- Martin, J. H. & Fitzwater, S. E. *Nature* **331**, 341–343 (1988).
- Price, N. M., Ahner, B. A. & Morel, F. M. M. *Limnol. Oceanogr.* **39**, 520–539 (1994).
- Coale, K. H., Fitzwater, S. E., Gordon, R. M., Johnson, K. S. & Barber, R. T. *Nature* **379**, 621–624 (1996).
- Fuhrman, J. A., Sleeter, T. D., Carlson, C. A. & Proctor, L. M. *Mar. Ecol. Progr. Ser.* **57**, 207–217 (1989).
- Kirchman, D. L., Keil, R. G., Simon, M. & Welschmeyer, N. A. *Deep-Sea Res.* **40**, 967–988 (1993).
- Williams, P. J. le B. *Kiel. Meeresforsch.* **5**, 1–28 (1981).
- Price, N. M. et al. *Biol. Oceanogr.* **6**, 443–461 (1988).
- Maldonado, M. T. & Price, N. M. *Mar. Ecol. Progr. Ser.* (in the press).
- Brand, L. E. *Limnol. Oceanogr.* **36**, 1755–1771 (1991).
- Martin, J. H., Fitzwater, S. E. & Broenkow, W. W. *Deep-Sea Res.* **36**, 649–680 (1989).
- Martin, J. H. & Knauer, G. A. *Geochim. Cosmochim. Acta* **37**, 1639–1653 (1973).
- Booth, B. C., Lewin, J. & Lorenzen, C. J. *Mar. Biol.* **98**, 287–298 (1988).
- Miller, C. B. et al. *Limnol. Oceanogr.* **36**, 1600–1615 (1991).
- LaRoche, J., Boyd, P. W., McKay, R. M. L. & Gelder, R. J. *Nature* **382**, 802–805 (1996).
- Reid, R. T., Live, D. H., Faulkner, D. J. & Butler, A. *Nature* **366**, 455–548 (1993).

- Rue, E. L. & Bruland, K. W. *Mar. Chem.* **50**, 117–138 (1995).
- Brand, L. E., Sunda, W. G. & Guillard, R. R. L. *Limnol. Oceanogr.* **28**, 1182–1198 (1983).
- Sunda, W. G., Swift, D. G. & Huntsman, S. A. *Nature* **351**, 55–57 (1991).
- Ingram, J. L., Maaløe, O. & Neidhardt, F. C., *Growth of the Bacterial Cell* (Sinauer, Sunderland, MA, 1983).
- Rainnie, D. J. & Bragg, P. D. J. *Gen. Microbiol.* **77**, 339–349 (1973).
- Kirchman, D. L. *Mar. Ecol. Progr. Ser.* **62**, 47–54 (1990).
- Hudson, R. J. & Morel, F. M. M. *Limnol. Oceanogr.* **34**, 1113–1120 (1989).
- Lee, S. & Fuhrman, J. A. *Appl. Environ. Microbiol.* **53**, 1298–1303 (1987).
- Booth, B. C., Lewin, J. & Postel, J. R. *Prog. Oceanogr.* **32**, 57–99 (1993).
- Neuer, S. *Mar. Ecol. Progr. Ser.* **83**, 251–262 (1992).
- Welschmeyer, N., Goericke, R., Strom, S. & Peterson, W. *Limnol. Oceanogr.* **36**, 1631–1649 (1991).
- Packard, T. T. & Williams, P. J. le B. *Oceanol. Acta* **4**, 351–358 (1981).

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## Engrailed-1 as a target of the Wnt-1 signalling pathway in vertebrate midbrain development

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**A SECRETED signalling molecule encoded by the *Wnt-1* gene is required for development of the central nervous system. In mouse embryos homozygous for a *Wnt-1*-null allele, the midbrain and anterior hindbrain fail to develop<sup>1,2</sup>. This corresponds to the region where two transcription factors encoded by the mouse *engrailed* genes (*En-1* and *En-2*) are normally expressed<sup>3,4</sup>. Studies of the *Drosophila* orthologue of *Wnt-1*, *wingless*, indicate that *Wingless* signal is required to maintain *engrailed* expression in neighbouring cells of the developing epidermis<sup>5–7</sup>. Here we report that expression of *En-1* in the developing midbrain of *Wnt-1*-null embryos is sufficient to rescue early midbrain and anterior hindbrain development. This suggests that a key role of *Wnt-1* signalling is to maintain *En* expression and that this aspect of the *Wnt-1*/*engrailed* interaction has been conserved from flies to mice.**

Genetic analysis of *Drosophila* development indicates that *Wingless* signalling in the epidermis is required for the maintenance of *engrailed* expression in adjacent cells<sup>5–9</sup>. In the mouse, *Wnt-1* and *Engrailed-1* (*En-1*) are first expressed in the presump-

tive midbrain, from ~8.0 days post coitum (d.p.c.) and continue to be expressed, together with *En-2*, in overlapping patterns during midbrain development<sup>3,4,10</sup>. In *Wnt-1*<sup>-/-</sup> (*Wnt-1*-null) embryos, *En-1* and *En-2* expression is initiated normally, but subsequently both domains of *En* expression are lost, which is concomitant with a failure of midbrain and anterior hindbrain development<sup>4,11</sup>. Although neither single *En* mutant has as severe a phenotype<sup>12,13</sup>, compound mutants have a similar midbrain and anterior hindbrain phenotype to that of *Wnt-1*<sup>-/-</sup> mutants (W. Wurst and A. Joyner, personal communication). Together these results suggest that *Wnt-1* signalling may regulate brain development by maintaining *En* transcription. If this is so, then maintaining midbrain expression of *En-1* should rescue the *Wnt-1*-null phenotype. To test this, we have used a *Wnt-1* regulatory element to express *En-1* in *Wnt-1*<sup>-/-</sup> embryos.

*En-1* was expressed from the transgene *WEXPZ-En-1* (ref. 14) in a pattern similar to that of endogenous *Wnt-1* gene (Fig. 1a–e)<sup>4,15</sup>. To assess whether *En-1* was able to rescue the *Wnt-1*-null phenotype, embryos from matings of *Wnt-1*<sup>+/-</sup>; *WEXPZ-En-1*<sup>+</sup> males with *Wnt-1*<sup>+/-</sup> females were collected at 14.5 d.p.c., when the *Wnt-1*<sup>-/-</sup> phenotype can easily be scored morphologically. The genotype was subsequently determined by Southern blotting (Fig. 1f, g). *Wnt-1*<sup>+/+</sup> and *Wnt-1*<sup>+/-</sup> embryos with or without *WEXPZ-En-1* appeared to be wild-type (*n* = 112) whereas all *Wnt-1*<sup>-/-</sup> embryos (*n* = 12) displayed the *Wnt-1*<sup>-/-</sup> phenotype. In *Wnt-1*<sup>-/-</sup>; *WEXPZ-En-1*<sup>+</sup> embryos, 7 out of 17 appeared superficially wild-type, 8 out of 17 were partially rescued and only 2 out of 17 were similar to *Wnt-1*<sup>-/-</sup> embryos.

To characterize brain development in greater detail, a minimum of four embryos from each category were sectioned for histological analysis. All *Wnt-1*<sup>-/-</sup> embryos lacked the midbrain and cerebellum (Fig. 2b), as described previously<sup>1</sup>. In contrast, in *Wnt-1*<sup>-/-</sup>; *WEXPZ-En-1*<sup>+</sup> embryos that were scored as wild-type, the midbrain and cerebellum appeared similar to those of

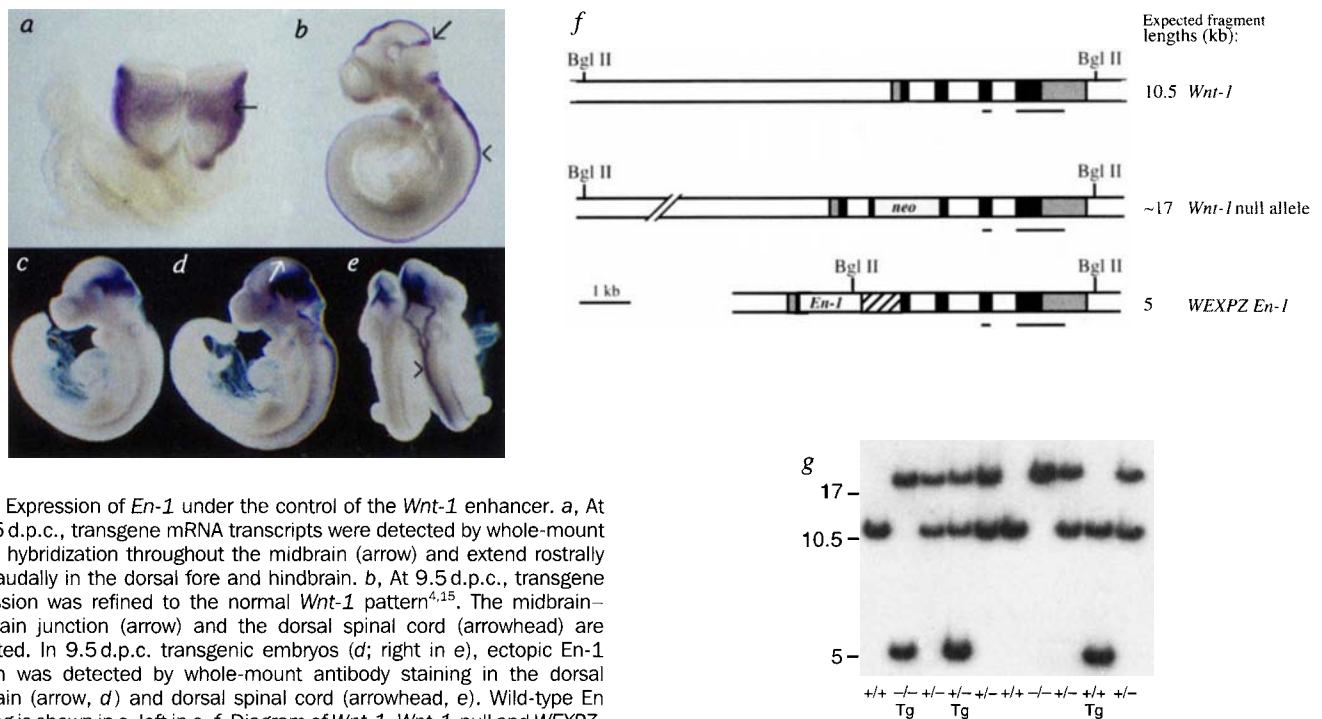


FIG. 1 Expression of *En-1* under the control of the *Wnt-1* enhancer. a, At ~8.25 d.p.c., transgene mRNA transcripts were detected by whole-mount *in situ* hybridization throughout the midbrain (arrow) and extend rostrally and caudally in the dorsal fore and hindbrain. b, At 9.5 d.p.c., transgene expression was refined to the normal *Wnt-1* pattern<sup>4,15</sup>. The midbrain–hindbrain junction (arrow) and the dorsal spinal cord (arrowhead) are indicated. In 9.5 d.p.c. transgenic embryos (d; right in e), ectopic *En-1* protein was detected by whole-mount antibody staining in the dorsal midbrain (arrow, d) and dorsal spinal cord (arrowhead, e). Wild-type *En* staining is shown in c, left in e. f, Diagram of *Wnt-1*, *Wnt-1*-null and *WEXPZ-En-1* loci (see Methods). The probe used for genotyping is represented by the broken line. g, Representative Southern blot of yolk-sac DNA from embryos derived from mating *Wnt-1*<sup>+/-</sup>; *WEXPZ-En-1*<sup>+</sup> males with *Wnt-1*<sup>+/-</sup> females. Tg, transgene.

wild-type embryos (compare Fig. 2a with 2c). In partially rescued embryos, only the posterior midbrain and a slightly reduced cerebellum were apparent (Fig. 2d). The absence of rescue in some cases, and partial rescue in others, may reflect influences of the genetic background or variations in the levels of *En-1* expressed from the transgene.

To characterize the development of the midbrain in *Wnt-1*<sup>-/-</sup>; *WEXPZ-En-1*<sup>+</sup> embryos further, the expression of several genes normally transcribed in this region was examined at 10.5 d.p.c. *Pax-5* is expressed in a broad domain at the midbrain–hindbrain junction<sup>16</sup> (Fig. 3a), but this domain is missing in *Wnt-1*<sup>-/-</sup> embryos (Fig. 3b). In *Wnt-1*<sup>-/-</sup>; *WEXPZ-En-1*<sup>+</sup> embryos, *Pax-5* expression was detected in a pattern similar to that of the wild-type embryos (Fig. 3c). *Wnt-1* and *Fgf-8* are normally expressed in adjacent rings of cells just anterior and posterior to the midbrain–hindbrain junction, respectively<sup>15,17,18</sup> (Fig. 3d, g). FGF8 signalling is known to be important in midbrain development<sup>19</sup>. In *Wnt-1*<sup>-/-</sup> embryos, both rings of expressing cells are absent (Fig. 3e, h). In contrast, both *Wnt-1* and *Fgf-8* were expressed in sharp rings of cells in *Wnt-1*<sup>-/-</sup>; *WEXPZ-En-1*<sup>+</sup> embryos (Fig. 3f, i). This was despite the fact that no morphologically obvious midbrain–hindbrain junction was apparent. These results suggest that *Wnt-1* signalling at this later stage may not play a direct role in regulating *Fgf-8* expression in adjacent cells. *En* gene expression was also restored in the mid-hindbrain region of *Wnt-1*<sup>-/-</sup>; *WEXPZ-En-1*<sup>+</sup> embryos outside the area where the transgene is expressed (compare Fig. 3f with i).

In all the rescued embryos, the expression domains of *Pax-5*, *Fgf-8*, *En*, and, in a few cases, *Wnt-1* were slightly reduced relative to wild-type littermates (18 out of 41 *Wnt-1*<sup>-/-</sup>; *WEXPZ-En-1*<sup>+</sup> embryos expressed one of the markers examined; of these at least half were substantially rescued). One likely explanation is that rescued embryos have a smaller population of midbrain cells than wild-type siblings because when *Wnt-1* and *En-1* expression is initiated, *Wnt-1* mRNA transcription is patchy, whereas *En* genes are expressed more uniformly in presumptive midbrain cells<sup>4,20</sup>. Thus in *Wnt-1*<sup>-/-</sup>; *WEXPZ-En-1*<sup>+</sup> embryos, where *En-1* is not

uniformly expressed in all presumptive midbrain cells, only those cells that express *En-1* at this early stage may contribute to midbrain development. As *En-1* expression in the midbrain restores *Fgf-8*, *Pax-5* and *En* expression in the anterior hindbrain, and subsequently cerebellum development in *Wnt-1*<sup>-/-</sup> embryos, we suggest that a midbrain-derived signal other than *Wnt-1* is necessary for anterior hindbrain development.

To assess whether expression of *En-1* was sufficient to rescue the viability of *Wnt-1*<sup>-/-</sup> mice (pups are born but die within 24 h;

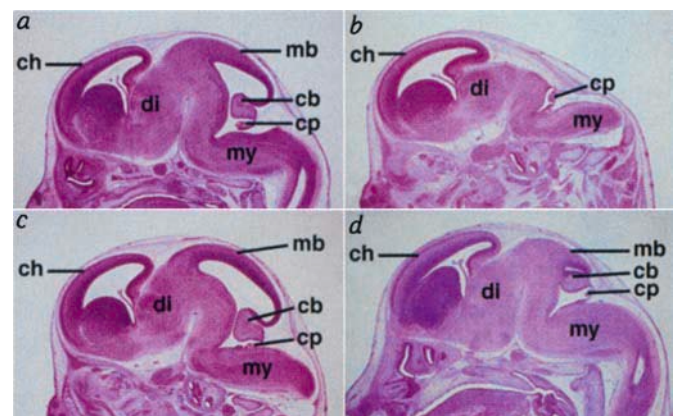


FIG. 2 Histological analysis of CNS development in *Wnt-1*<sup>-/-</sup> embryos expressing *En-1* at 14.5 d.p.c. Sagittal views of a, *Wnt-1*<sup>+/+</sup>; b, *Wnt-1*<sup>-/-</sup>; and c, d, *Wnt-1*<sup>-/-</sup>; *WEXPZ-En-1*<sup>+</sup> embryos. The posterior choroid plexus (cp) marks the metencephalic–myelencephalic junction. Embryos that were *Wnt-1*<sup>+/+</sup> or *Wnt-1*<sup>-/-</sup> with or without the transgene were indistinguishable at this stage. Ch, cerebral hemisphere; di, diencephalon; mb, midbrain; cb, cerebellum; cp, choroid plexus; my, myelencephalon (caudal hindbrain).



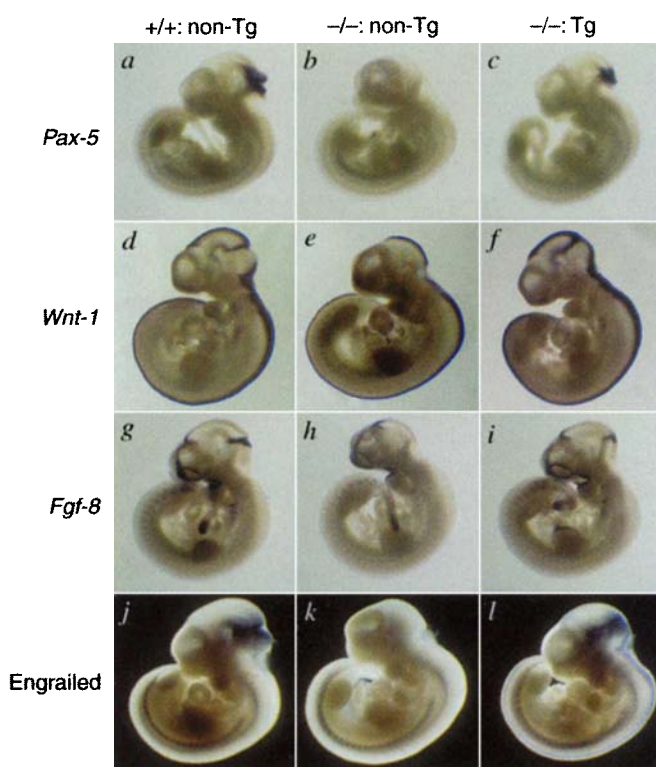


FIG. 3 Expression of midbrain-hindbrain markers in 10.5 d.p.c. embryos. *Pax-5* (a–c), *Wnt-1* (d–f) and *Fgf-8* (g–i) expression was visualized by whole-mount *in situ* hybridization and both *En-1* and *En-2* expression (j–l) by antibody staining. Representative examples of wild-type (a, d, g, j; +/+; non-Tg) and *Wnt-1* mutants (b, e, h, k; –/–; non-Tg) which do not carry the transgene and *Wnt-1* mutants carrying the transgene (c, f, i, l; –/–; Tg) are shown. No alteration in the normal expression pattern of any of the markers was observed in wild-type embryos expressing the transgene.

ref. 1) pups were genotyped at 10 days post partum ( $n = 68$ ), but no live *Wnt-1*<sup>–/–</sup>; *WEXPZ-En-1*<sup>+</sup> mice were obtained, so *En-1* was insufficient to rescue the *Wnt-1*-null phenotype completely. Further analysis indicated that between 14.5 and 18.5 d.p.c., brains of *Wnt-1*<sup>–/–</sup>; *WEXPZ-En-1*<sup>+</sup> embryos deteriorate (data not shown). Thus there may be additional functions of *Wnt-1* signalling that cannot be replaced by *En-1*. This conclusion is supported by analysis of two cranial motor nerves, III (oculomotor) and IV (trochlear), which normally develop adjacent to *Wnt-1*-expressing cells in the ventral midbrain. Each of these fail to develop in *Wnt-1*<sup>–/–</sup> embryos<sup>21</sup>. Similarly, neither nerve forms in *Wnt-1*<sup>–/–</sup>; *WEXPZ-En-1*<sup>+</sup> embryos which have global restoration of midbrain development (data not shown). In contrast, we have found that a second ventral population, tyrosine-hydroxylase-expressing neurons (catecholaminergic neurons) of the substantia nigra are rescued in *Wnt-1*<sup>–/–</sup>; *WEXPZ-En-1*<sup>+</sup> embryos (data not shown).

In summary, in the absence of a *Wnt-1* signal, expression of *En-1* from the *Wnt-1* enhancer is sufficient to substantially rescue early midbrain and anterior hindbrain development. These results suggest that a major role of *Wnt-1* signalling in the mammalian brain is to maintain *En* expression. This is identical to a role proposed for Wingless signalling in the *Drosophila* epidermis, demonstrating that this aspect of the regulatory pathway has been conserved from flies to mice. □

## Methods

**Transgenic mice.** The expression vector pWEXPZ-En-1 contains the *En-1* cDNA and an 825-base-pair piece of *E. coli lacZ* as an mRNA tag (hatched box in Fig. 1f) inserted at the *EcoRV* site of pWEXP2 (ref. 14). Filled and shaded boxes in Fig. 1f represent translated and untranslated parts of *Wnt-1* exons,

respectively. Transgenic lines were generated essentially as described<sup>14</sup>. Nine transmitting founder lines were generated, three of which expressed the transgene appropriately in embryos. These were crossed with *Wnt-1*<sup>+/–</sup> mice<sup>1</sup> to generate *Wnt-1*<sup>+/–</sup>; *WEXPZ-En-1*<sup>+</sup> males. Expression of *En-1* from the transgene resulted in severe hydrocephalus and lethality in three founder mice and in transgenic offspring of two expressing founders (data not shown) but one line was stably maintained on the *Wnt-1*<sup>+/–</sup> background. Southern blotting of yolk-sac DNA was done described<sup>22</sup> using the probe described<sup>1</sup>.

**Whole-mount *in situ* hybridization.** This was done with digoxigenin-labelled probes as described<sup>15</sup> and modified according to ref. 23. *Pax-5*<sup>16</sup>, *Wnt-1*<sup>15</sup> and *Fgf-8*<sup>18</sup> probes have been described. The probe used to detect *Wnt-1* mRNA also detects transgene mRNA. The transgene-specific probe was generated from the *lacZ* tag in pWEXPZ-En-1.

**Whole-mount antibody staining.** Expression of *En-1* and *En-2* proteins was detected by whole-mount antibody staining using the polyclonal antiserum  $\alpha$ Enhb-1, which recognizes both proteins<sup>20</sup>.

**Histological analysis.** Embryos were collected in PBS, fixed in Bouin's fixative, dehydrated, embedded in wax and sectioned at 6  $\mu$ m. Sections were dewaxed, rehydrated and stained with haematoxylin and eosin.

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- McMahon, A. P. & Bradley, A. *Cell* **62**, 1073–1085 (1990).
- Thomas, K. R. & Capecchi, M. R. *Nature* **346**, 847–850 (1990).
- Davis, C. A. & Joyner, A. L. *Genes Dev.* **2**, 1736–1744 (1988).
- McMahon, A. P., Joyner, A. L., Bradley, A. & McMahon, J. A. *Cell* **69**, 581–595 (1992).
- DiNardo, S., Sher, E., Heemskerk-Jongens, J., Kassir, J. & O'Farrell, P. *Nature* **332**, 604–609 (1988).
- Martinez-Arias, A., Baker, N. E. & Ingham, P. W. *Development* **103**, 157–170 (1988).
- Heemskerk, J., DiNardo, S. & Kostriken, R. *Nature* **352**, 404–410 (1991).
- Ingham, P. W. & Martinez-Arias, A. *Cell* **68**, 221–235 (1992).
- Klingensmith, J. & Nusse, R. *Dev. Biol.* **170**, 636–650 (1995).
- Rowitch, D. H. & McMahon, A. P. *Mech. Dev.* **52**, 3–8 (1995).
- Mastick, G. S. et al. *J. Comp. Neurol.* (in the press).
- Wurst, W., Auerbach, A. B. & Joyner, A. L. *Development* **120**, 2065–2075 (1994).
- Joyner, A. L. et al. *Science* **251**, 1239–1243 (1991).
- Echelard, Y. et al. *Cell* **75**, 1417–1430 (1993).
- Parr, B., Shea, M., Vassileva, G. & McMahon, A. P. *Development* **119**, 247–261 (1993).
- Asano, M. & Gruss, P. *Mech. Dev.* **39**, 29–39 (1992).
- Crossley, P. H. & Martin, G. R. *Development* **121**, 439–451 (1995).
- Mahmood, R. et al. *Curr. Biol.* **5**, 797–806 (1995).
- Crossley, P. H., Martinez, S. & Martin, G. R. *Nature* **380**, 66–68 (1996).
- Davis, C. A., Holmyard, D. P., Millen, K. J. & Joyner, A. L. *Development* **111**, 287–298 (1991).
- Fritsch, B., Nichols, D. H., Echelard, Y. & McMahon, A. P. *J. Neurobiol.* **27**, 457–469 (1995).
- Takada, S. et al. *Genes Dev.* **8**, 174–189 (1994).
- Knecht, A. K., Good, P. J., Dawid, I. B. & Harland, R. M. *Development* **121**, 1927–1936 (1995).

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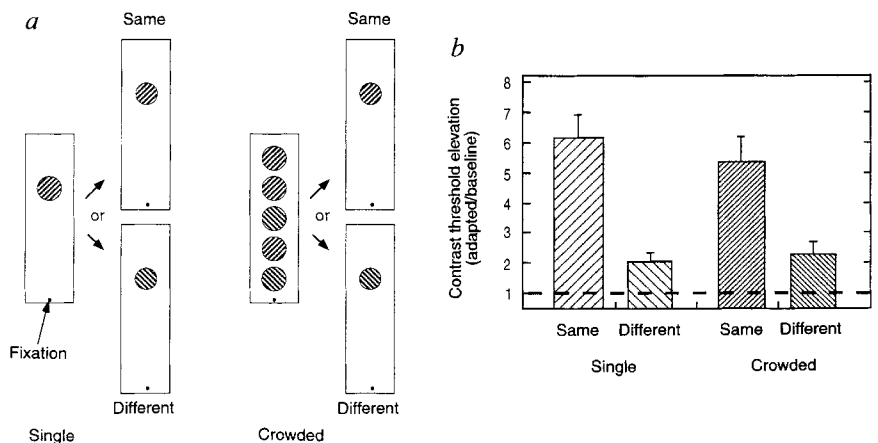
## Attentional resolution and the locus of visual awareness

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**VISUAL spatial resolution is limited by factors ranging from optics to neuronal filters in the visual cortex<sup>1,2</sup>, but it is not known to what extent it is also limited by the resolving power of attention. To investigate this, we studied adaptation to lines of specific orientation, a process that occurs in primary visual cortex<sup>3</sup>. When a single grating is presented in the periphery of the visual field, human observers are aware of its orientation, but when it is flanked by other similar gratings ('crowding'), its orientation becomes impossible to discern<sup>4,5</sup>. Nevertheless, we show that orientation-specific adaptation is not affected by crowding, implying that spatial resolution is limited by an attentional filter acting beyond the primary visual cortex. Consistent with this, we find that attentional resolution is greater in the lower**

FIG. 1 Orientation-selective adaptation when subjects could and could not perceive the orientation of the adapting grating (fourth grating from the fixation point). In all experiments, stimuli were circular patches of sinusoidal gratings. *a*, Contrast thresholds were measured for three conditions: adapted to a single grating; adapted to the fourth stimulus in a linear array of five; no adaptation (baseline). The adapting grating and test grating were centred  $25^\circ$  above the point of visual fixation. The radius for each grating was  $2.67^\circ$ , and the centre-to-centre distance between neighbouring gratings was  $6.67^\circ$ . Except in the baseline condition, subjects were exposed to a continuous 5-s adaptation, 300-ms interstimulus interval, 180-ms test cycle, during which threshold contrast for the test grating was set by the subjects. Settings were not recorded during the first 10 cycles to ensure that subjects could achieve a stable adapted state. For each adapting-testing cycle, the orientation of the first grating was randomly chosen to be either  $45^\circ$  or  $135^\circ$ , and the orientation of the other distractors were successively rotated by  $90^\circ$ . Orientation of the fourth target grating was predetermined and remained the same for each session. *b*, Threshold contrast elevation after adaptation, compared with baseline contrast threshold before adaptation. Mean of four subjects; error bars show s.e.m. The difference between



same adapt-test orientation and different adapt-test orientation represents the strength of orientation selective adaptation. The data show that a flanked grating (orientation not perceivable) and a single grating (orientation readily perceivable) were almost equally effective in orientation-specific adaptation.

than in the upper visual field, whereas there is no corresponding asymmetry in the primary visual cortex. We suggest that the attentional filter acts in one or more higher visual cortical areas to restrict the availability of visual information to conscious awareness<sup>6</sup>.

A high-contrast adapting grating patch of one cycle per degree was presented  $25^\circ$  above the point of visual fixation. When presented alone, the orientation of the grating was clearly visible even at contrasts of less than 10%. When the same adapting grating was presented fourth in a linear array of five similar grating patches (maximizing the crowding effect<sup>5,7</sup>), subjects identified the orientation at chance levels, even with unlimited viewing time at full contrast (Fig. 1a). We tested whether there was any orientation-specific analysis of the grating when the subjects were unaware of the orientation. Observers were adapted to a target grating presented alone or with flanking gratings (one above and three below the target), and then were tested on their ability to identify the orientation of a grating either at the same or orthogonal orientation presented at the target position (Fig. 1a). When observers were adapted to a crowded grating (and were unaware of its orientation), the contrast thresholds in tests in which the grating was of the same orientation as the adapting grating were roughly 2 to 3 times higher than for tests using the orthogonal orientation (see Fig. 1b). We found this same orientation-specific threshold elevation when observers were adapted to

a single grating, and were aware of its orientation. There is a clear dissociation between perceptual awareness of orientation and orientation-specific adaptation (located at or beyond the primary visual cortex (area V1), the first site of orientation processing). The results indicate that visual awareness, and the 'crowding' that blocks it, occur after orientation analysis in the visual information processing stream. Others have reported that unresolvable high-frequency gratings can produce orientation-specific adaptation<sup>2</sup>, and orientation information can be used for segmentation without the explicit knowledge of the subject<sup>8</sup>. Our results indicate further that activation of neurons in V1 is insufficient for conscious perception<sup>6</sup>.

Conventionally, the reduced sensitivity to targets embedded in a field of similar items is termed 'lateral masking' and is often attributed to lateral inhibition between neighbouring neurons at early stages of processing<sup>9</sup>. We show that the information that is inaccessible to perceptual awareness because of crowding, is in fact processed by the primary visual cortex without disruption, ruling out the simplest sensory lateral masking explanation. We believe that this crowding effect reflects the limited resolution of the spatial attention mechanism.

The crowding effect seemed stronger in the upper visual field than in the lower field. Lesion<sup>10</sup> and functional magnetic resonance imaging (fMRI)<sup>11,12</sup> studies show that human primary visual cortex devotes roughly the same area to the representations of the

FIG. 2 Discrimination of grating orientation. *a*, Examples of four stimulus conditions. All gratings were 1 cycle per degree. Grating patches had a  $2^\circ$  radius, and the centre of the target grating was  $20^\circ$  from the point of fixation. In the crowding condition, the centre-to-centre distance between neighbouring gratings was  $5^\circ$ . Stimuli were presented for 180 ms. Subjects were instructed to always report (and hence attend to) only the orientation of the grating patch in the middle, ignoring the flanking gratings when they appeared. Visual field positions were fixed during each session, and varied between sessions. Stimuli were presented at 4 different contrast levels (target and flanking stimuli always had the same contrast). *b*, Mean  $\pm$  s.e.m. accuracy from three subjects. Symbols correspond to the conditions depicted in *a*. Whereas subjects could see the grating orientation almost equally well in both the upper and lower visual field (open symbols), their ability to identify the orientation of the target grating was much more affected by flanking stimuli when presented in the upper visual field (filled triangles) than in the lower visual field (filled circles).

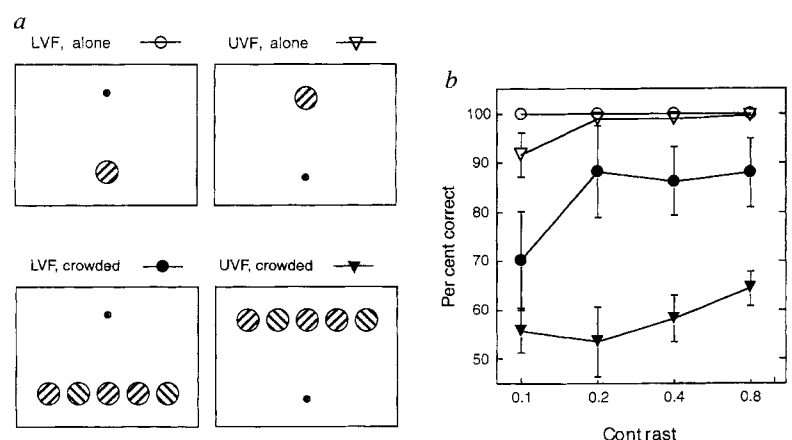
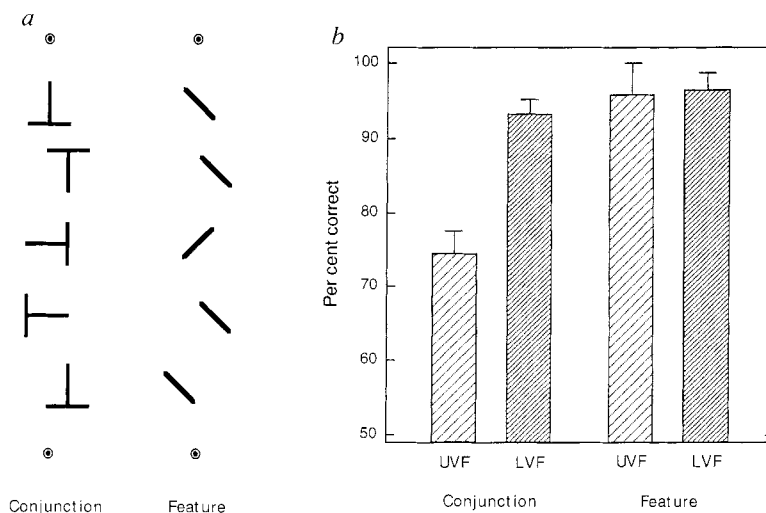


FIG. 3 *a*, Schematic examples of stimuli used in the feature detection and the conjunction detection condition. Subjects attended to the central stimulus, and responded to the presence or absence of the prespecified target (in this example, the 45° bar is the target in the feature case and the T tilted 90° clockwise is the conjunction target). Line segments in both cases subtended 2° and vertical centre-to-centre distance between elements was 3°. To reduce the contribution of global shape recognition on detection ability, the horizontal position of each element was randomly centred 1° to the left or right of the vertical midline. Stimuli were presented for 150 ms and feedback about whether the response was correct was given in each trial. *b*, Accuracy data collected in four conditions. Each bar is the mean  $\pm$  s.e.m. of 4 subjects. Conjunction detection suffers a large asymmetry between upper (UVF) and lower visual field (LVF), whereas feature detection is equally good in both the upper and lower visual field.



upper and lower visual fields. Consequently, we would not expect any asymmetry in crowding effects if performance were determined principally in the primary visual cortex. To test this prediction, we compared performance in the upper and lower visual fields directly in an orientation discrimination task. Rather than maximizing the crowding effect with a radial stimulus organization<sup>5,7</sup> as in the first experiment, we arranged the stimuli horizontally to reduce crowding and to expose any modulation by visual field. The stimulus consisted of either a single grating patch or the same grating patch flanked by four similar patches, two on each side (Fig. 2*a*). The target grating was tilted either to the right (45°) or to the left (135°). Subjects were asked to fixate a point above the stimulus pattern in half of the trials, and a point the same distance below it on the other half. The stimulus was presented briefly (180 ms), and subjects were asked to press one of two keys to indicate which way the central grating was tilted, guessing if necessary. The results (Fig. 2*b*) reveal a strong asymmetry in performance: crowding reduced the accuracy of report significantly more when the target was in the upper visual field than when it was in the lower field.

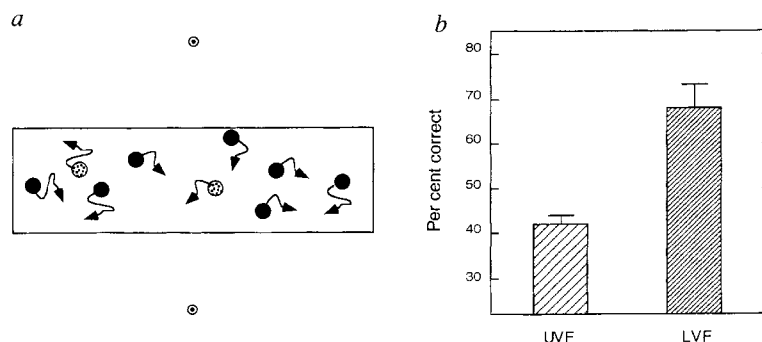
If crowding is a result of insufficient spatial resolution of attention, then performance in tasks that require more focused attention should be better when the stimulus is presented in the lower visual field than in the upper visual field, whereas tasks that demand little focused attention should show little or no asymmetry. Visual search tasks provide examples of both attentional categories. Many studies have shown that searches for feature-defined targets (where the target has the odd feature in an otherwise homogeneous array) require little focused attention, whereas searches for conjunction targets (where targets and distractors share features but differ in how they are combined) often require focused attention<sup>13</sup>. We used two simplified versions of these tasks. Five items were presented along a vertical line

above or below the point of visual fixation. In half of the trials, the item in the centre was the prespecified target, and in the other trials, the central item was a distractor. In the feature detection condition, the target was a line angled at 45° among 135° distractors, and in the conjunction detection condition, the target was a vertical and a horizontal line arranged in a 'T' shape rotated by 90° to the right, with distractors of the same shape in the three other orientations (upright, upside down, 90° rotation to the left). The target and distractors all contain the same features and are distinguished only by the conjunction of their positions (Fig. 3*a*). In the feature detection task, subjects performed equally well when targets were presented in the upper and lower visual field, whereas in the conjunction detection task, subjects showed the predicted, improved detection ability in the lower visual field (Fig. 3*b*).

Finally we used an attentional tracking task that places strong and constant demands on the attentional system<sup>14,15</sup>. Subjects were presented with 9 green discs moving randomly in a rectangular area in the upper or lower visual field. At the start of each trial, two discs were identified as targets by briefly turning red. They then turned back to green and subjects were asked to track the two targets with their attention while fixating a central dot. At the end of 5 s, all discs stopped moving and subjects were required to report which were the original targets. Tracking performance was significantly better in the lower visual field than in the upper visual field (Fig. 4).

Without distractors, perception of spatial details is limited by conventional visual resolution. However, when several items are presented, perception of the spatial details of a particular item seems to depend on the ability of attentional processes to isolate the item. This suggests that attentional resolution limits the access of spatial details to perceptual awareness. We believe that the frequently reported degradation of spatial information when

FIG. 4 Attentional tracking task and accuracy data. *a*, Subjects fixated 10° above or below the centre of a 6.6°  $\times$  30° area in which nine green moving discs were presented. At the start of each trial, two of the discs were changed to red for 1 s, and then turned back to green. Subjects were required to track these two discs with their attention while keeping their gaze steady on the fixation dot. After 5 s, all nine discs stopped moving, and subjects were asked to identify the target discs. Chance performance was 22.2%. *b*, Subjects performed much better when the discs were presented in the lower visual field. Mean  $\pm$  s.e.m. of 4 subjects.



several items are present in the visual field may be a result of this limitation<sup>16,17</sup>. The asymmetry in attentional resolution between the upper and lower visual field may also contribute to the reported lower visual field advantage for the perception of global shape in tasks involving hierarchical structures where several elements are used<sup>18–20</sup>.

We attempted to locate the cortical area that mediates visual attention and limits the final access to our conscious vision. We show that stimuli not available to conscious perception could produce undiminished orientation-specific adaptation, a process that first occurs in primary visual cortex. Clearly, neuronal activity in the primary visual cortex is not sufficient for conscious percep-

tion. This is further supported by the fact that although there is little anatomical asymmetry between the upper and lower visual fields in human primary visual cortex<sup>10–12</sup>, crowding and other manipulations of attentional load produced a large asymmetry favouring the lower visual field. The same holds for V2 as well. The dorsal parietal system has classically been associated with attentional processes<sup>21,22</sup> and the projections from early visual areas to the parietal regions are more numerous for the lower visual field than the upper field<sup>23</sup>. We suggest that the dorsal parietal area may control attentional resolution and the information entering our conscious vision. □

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- Campbell, F. W. & Gubisch, R. W. *J. Physiol.* **186**, 558–578 (1966).
- He, S., Smallman, H. S. & MacLeod, D. I. A. *Invest. Ophthalmol. Vis. Sci. Suppl.* **36**, 2010 (1995).
- Blakemore, C. B. & Campbell, F. W. *J. Physiol.* **203**, 237–260 (1969).
- Bouma, H. *Nature* **226**, 177–178 (1970).
- Toet, A. & Levi, D. M. *Vision Res.* **32**, 1349–1357 (1992).
- Crick, F. & Koch, C. *Nature* **375**, 121–123 (1995).
- Chambers, L. & Wolford, G. *Bull. Psychonom. Soc.* **21**, 459–461 (1983).
- Kolb, F. C. & Braun, J. *Nature* **377**, 336–338 (1995).
- Chastain, G. *Psychol. Res.* **45**, 147–156 (1983).
- Horton, J. C. & Hoyt, W. F. *Archives Ophthalmol.* **109**, 816–824 (1991).
- Sereno, M. I. et al. *Science* **268**, 889–893 (1995).
- DeYoe, E. A. et al. *Proc. Natl Acad. Sci. USA* **93**, 2382–2386 (1988).
- Treisman, A. Q. J. *Exp. Psychol. Human Exp Psychol.* **40**, 201–237 (1988).
- Polyshyn, Z. W. & Storm, R. W. *Spatial Vis.* **3**, 179–197 (1988).

- Intriligator, J., Nakayama, K. & Cavanagh, P. *Invest. Ophthalmol. Vis. Sci. Suppl.* **32**, 1040 (1991).
- Butler, B. E. & Currie, A. *Psychol. Res.* **48**, 201–209 (1986).
- Taylor, S. G. & Brown, D. R. *Percept. Psychophys.* **12**, 97–99 (1972).
- Previc, F. H. *Behav. Brain Sci.* **13**, 519–575 (1990).
- Christman, S. D. *Bull. Psychonom. Soc.* **31**, 275–278 (1993).
- Rubin, N., Nakayama, K. & Shapley, R. *Science* **271**, 651–653 (1996).
- Gazzaniga, M. S. & Ladavas, E. in *Neurophysiological and Neuropsychological Aspects of Spatial Neglect* (ed. Jeannerod, M.) 203–213 (Elsevier, Amsterdam, 1987).
- Posner, M. I. *Neuropsychol. Rehab.* **4**, 183–187 (1994).
- Maunsell, J. H. & Newsome, W. T. *Annu. Rev. Neurosci.* **10**, 363–401 (1987).

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## Homodimeric architecture of a ClC-type chloride ion channel

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THE recent discovery of the ClC-family of anion-conducting channel proteins<sup>1–3</sup> has led to an appreciation of the central roles played by chloride ion channels in cellular functions, such as electrical behaviour of muscle<sup>4–7</sup> and nerve<sup>8</sup> and epithelial solute transport<sup>9</sup>. Little is known, however, about molecular architecture or sequence–function relationships in these membrane proteins. In the single case of ClC-0, a voltage-gated ‘muscle-type’ chloride channel, the functional complex is known to be a homo-oligomer of a polypeptide of  $M_r \sim 90,000$ , with no associated ‘helper’ subunits<sup>10</sup>. The subunit stoichiometry of ClC-type channels is controversial, however, with either dimeric or tetrameric association suggested by different indirect experiments<sup>10,11</sup>. Before a coherent molecular view of this new class of ion channels can emerge, the fundamental question of subunit composition must first be settled. We have examined hybrid ClC-0 channels constructed from functionally tagged subunits, and report here that ClC-0 is a homodimer containing two chloride-conduction pores.

These experiments examine ClC-0 channels purified and functionally reconstituted after high-level heterologous expression in mammalian cells. The expressed channels display the same molecular and functional characteristics observed<sup>10</sup> with ClC-0 purified from its natural source, *Torpedo* electroplax (Fig. 1). Both preparations run identically on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) gels as single, lightly glycosylated 90K bands; the expressed, purified channels incorporated into planar lipid bilayers show the same single-channel conductance and voltage-dependent gating behaviour

as the native *Torpedo* channel<sup>12–14</sup>. An unusual characteristic of ClC-0 channels—and one essential to grasp for addressing questions of molecular structure—is the existence of two identical and physically separate Cl<sup>–</sup>-conduction pores in a ‘double-barrelled’ complex<sup>12–16</sup>. This feature is manifested in the single-channel records by three distinct, equally spaced conductance levels, in which the pores are either both closed (level 0), one open and one closed (level 1), or both open (level 2). Because the two pores are identical, level 1 is degenerate, the conductance being the same regardless of which pore is open.

Recently, residue K519, putatively located on the cytoplasmic side of the membrane just after the twelfth transmembrane domain, was found by macroscopic electrophysiological experiments to influence gating and anion permeation<sup>17</sup>. The single-pore conductance is sensitive to the charge at this position (Fig. 2). Similar conductances are seen for K519 ( $16.1 \pm 1$  pS) and R519 ( $13.1 \pm 0.3$  pS), whereas electrically neutral substitutions, regardless of their chemical nature (C519, Q519, M519, F519), yield channels with about half this conductance (5–7 pS). Substitution of a negative residue lowers the conductance further (2–3 pS). The dependence of conductance on side-chain charge argues that the residue influences Cl<sup>–</sup> permeation primarily by an electrostatic mechanism. A small but statistically significant effect of side-chain volume may also operate because the R519 conductance is lower than that of K519 and the F519 conductance falls below those of the smaller neutral replacements. All these charge-substituted channels display the three binomially distributed gating levels of the double-barrelled channel complex<sup>12,13</sup>, in which level 2 has precisely twice the conductance of level 1. The preferential effect of charge substitutions on inward current (Fig. 2b and ref. 17) indicates that residue 519 might be located on the cytoplasmic side of the channel, a weak suggestion that we unambiguously confirm below.

The single-channel conductance can be used as a functional tag in subunit-mixing experiments to deduce the number of ClC-0-519 residues contributing to the pore, and hence the number of subunits in the homo-oligomer. We coexpressed complementary DNAs coding for K519 and C519, purified the resulting channels, and examined the single-channel permeation properties of these



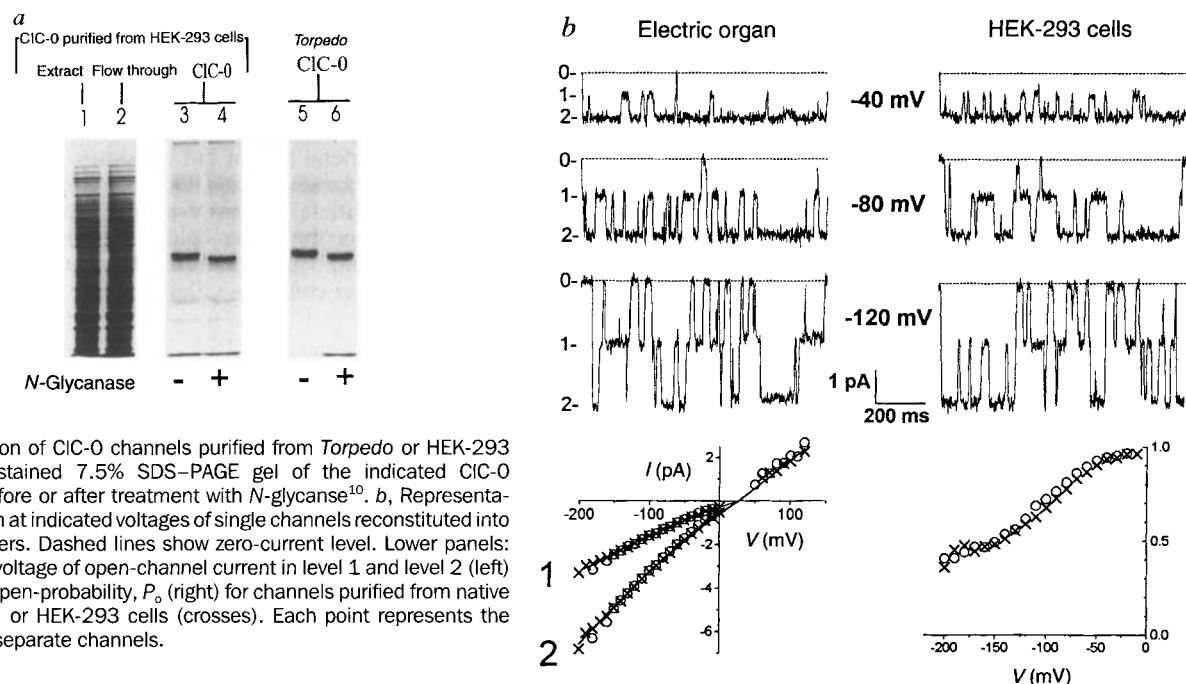


FIG. 1 Comparison of CIC-0 channels purified from *Torpedo* or HEK-293 cells. *a*, Silver-stained 7.5% SDS-PAGE gel of the indicated CIC-0 preparations, before or after treatment with *N*-glycanase<sup>10</sup>. *b*, Representative records taken at indicated voltages of single channels reconstituted into planar lipid bilayers. Dashed lines show zero-current level. Lower panels: dependence on voltage of open-channel current in level 1 and level 2 (left) or steady-state open-probability,  $P_o$  (right) for channels purified from native *Torpedo* (circles) or HEK-293 cells (crosses). Each point represents the average of 3–5 separate channels.

hybrids. These experiments optimized yields of hybrid channels by exploiting the opportunities offered by the immunoaffinity purification procedure. In K519, the natural 'RM2' epitope<sup>10</sup> was replaced with a haemagglutinin (HA) epitope, a manoeuvre that did not affect channel function (data not shown), whereas in C519 channels the RM2 epitope was preserved. In a coexpressed mixture, we could thus purify only C519 homo-oligomers and C519–K519 hetero-oligomers, while discarding the wild-type K519 homo-oligomers, as illustrated in Fig. 3a. Western blots (not shown) confirm that the purified CIC-0 preparations carry both the RM2 and HA epitopes.

Upon reconstituting these preparations into planar lipid bilayers, we observed two types of single CIC-0 channels. Of 17 channels observed, four were indistinguishable in all respects from homomeric C519, and one behaved like a wild-type K519 homomer contaminating the preparation. In contrast, 12 displayed a striking hybrid behaviour never before observed in CIC channels. Rather than showing three conductance levels, these hybrid

channels exhibit four, labelled 0, 1C, 1K, and 2 in Fig. 3b. Level 0 is nonconducting, as with homomeric CIC-0. The intermediate states 1C and 1K have precisely the single-pore conductances of the corresponding homo-oligomeric channels C519 and K519, as is clear from the aligned amplitude histograms (Fig. 3b) and from the summary (Fig. 3c) of our compiled observations from three coexpressed channel preparations. Moreover, the level 2 conductance is the sum of the level 1C and 1K conductances, a result that provides additional evidence that these gating substates indeed represent two physically distinct  $\text{Cl}^-$ -conduction pores in parallel. The degeneracy of homomeric level 1 is broken in the hybrid channel, because now the two pores have different conductances. Thus, the hybrid CIC-0 complex viewed as a whole displays a qualitatively novel behaviour not seen in either parental form; each individual pore, however, carries the pure conductance phenotype of its parent. Figure 3c also indicates that this was the only hybrid phenotype observed. We did not discern any intermediate pore-conductances, as with the multiple hybrid

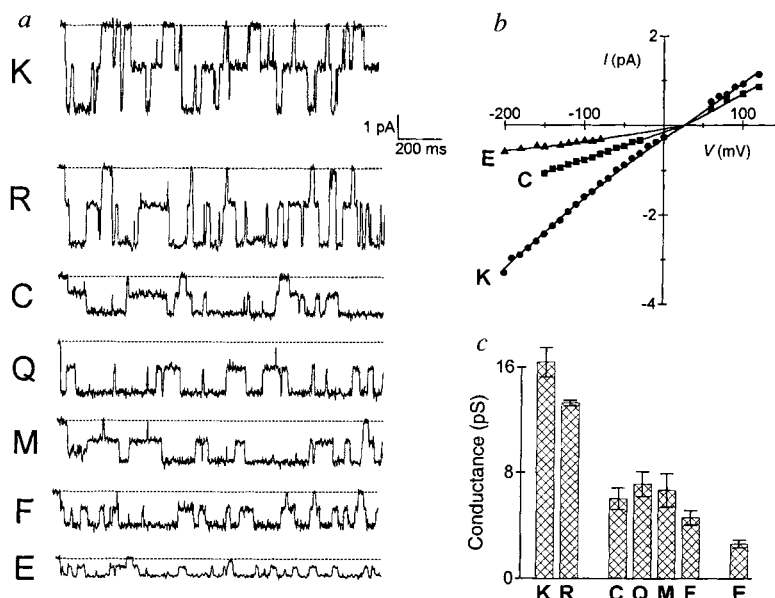


FIG. 2 Electrophysiological properties of CIC-0 channels with the indicated residues at position 519. CIC-0 channels with the indicated residues at position 519 were expressed in HEK-293 cells, purified and reconstituted into planar lipid bilayers. *a*, Single-channel recordings at -120 mV are shown for each CIC-0 variant. *b*, Single-pore  $I-V$  curves are shown for K519 (circles), C519 (squares) and E519 (triangles). *c*, Single-pore slope conductance (in the range -100 to -150 mV) of CIC-0 variants. Bars represent the means  $\pm$  s.d. of 2–9 determinations on separate channels. CIC-0 mutants were constructed by PCR-based cassette mutagenesis and verified by sequencing.

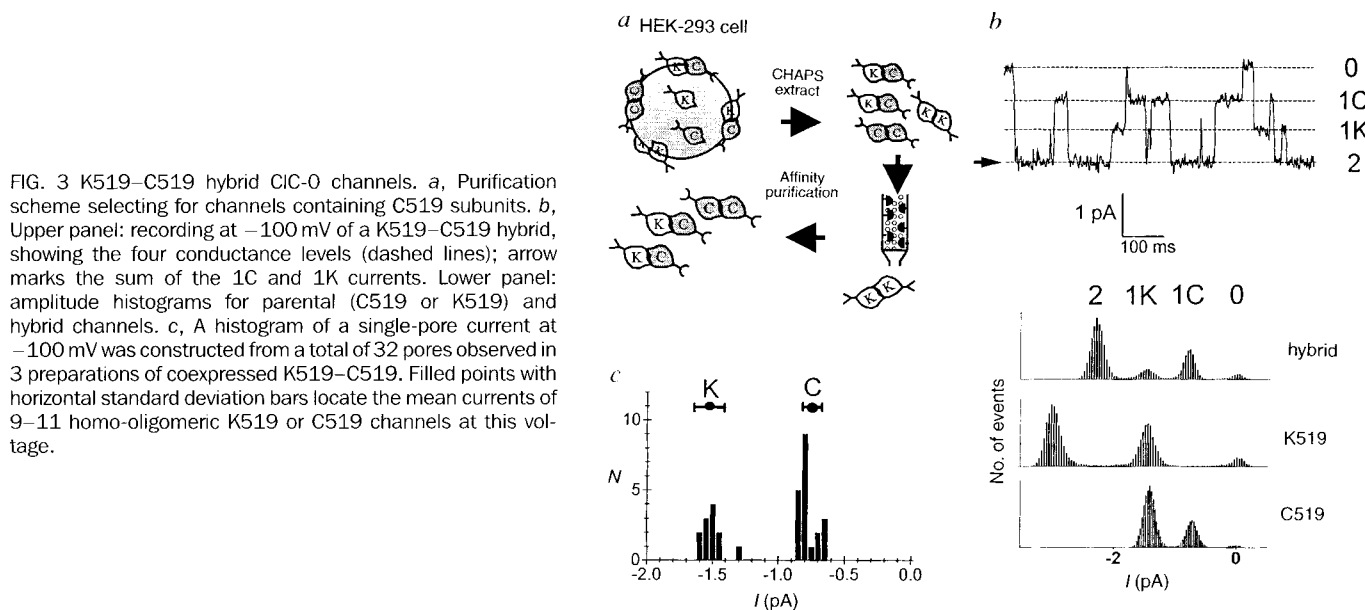


FIG. 3 K519-C519 hybrid CIC-O channels. **a**, Purification scheme selecting for channels containing C519 subunits. **b**, Upper panel: recording at  $-100$  mV of a K519-C519 hybrid, showing the four conductance levels (dashed lines); arrow marks the sum of the 1C and 1K currents. Lower panel: amplitude histograms for parental (C519 or K519) and hybrid channels. **c**, A histogram of a single-pore current at  $-100$  mV was constructed from a total of 32 pores observed in 3 preparations of coexpressed K519-C519. Filled points with horizontal standard deviation bars locate the mean currents of 9-11 homo-oligomeric K519 or C519 channels at this voltage.

pores that arise in coexpressed subunit mixtures of pentameric acetylcholine receptors<sup>18</sup> or tetrameric cyclic nucleotide-gated channels<sup>19</sup>. Therefore, the 519 residue manifests its influence only once in each pore.

We carried out additional subunit-counting experiments with homo-oligomeric C519 channels. The approach exploits the chemical reactivity of cysteine with methanethiosulphonate reagents<sup>20,21</sup> and the electrostatic influence of residue 519 on single-channel conductance. As outlined in Fig. 4a, we ask how many cysteine-modification events are required to convert the low-conductance C519 channel to the high-conductance phenotype expected from reaction with methanethiosulphonate ethylamine (MTSEA), which produces a positively charged side chain. We find that  $1 \mu\text{M}$  MTSEA added to the cytoplasmic side of C519 channels fully reverts the mutant channel to a high-conductance phenotype in a two-step reaction within 1-2 min (Fig. 4b). At the single-channel level, each of the two steps is individually observable. The first step (middle trace) converts one of the 6pS C519 pores to 13 pS, without affecting the other pore; following this first 'hit' by MTSEA, the channel displays four-level gating reminiscent of the coexpressed C519-K519 hybrids described above. About 30 s later, the remaining C519 pore is converted to a high-conductance pore by a second MTSEA hit (lower trace), and the channel again gates among only three levels. These conversion events were observed in all 19 attempts to react CIC-0-C519 channels with MTSEA.

Other charged MTS reagents alter the C519 pore as well (Fig. 4c). MTSET, a membrane-impermeant quaternary ammonium analogue of MTSEA, converts the C519 channel to a high-conductance phenotype in two discrete steps, but only when added to the cytoplasmic side of the channel; at 100-fold higher concentrations on the external side, MTSET fails to alter the C519 conductance, even after a 20-min exposure (data not shown). A negatively charged reagent bearing a sulphonate group, MTSES, converts the C519 channel to a low-conductance E519-like phenotype, also in two steps. The wild-type K519 channel does not show any pore modification by MTS reagents under similar conditions. A comparison of the altered channels (Fig. 4c) further suggests that the small effect of side-chain volume observed in the electrostatic point-mutants also applies to the 'unnatural' residues arising from the MTS reagents. The side-chain produced by MTSEA is slightly larger than lysine, and the conductance of the resulting pore is about 15% less than that of K519; a further 30% drop in conductance is brought about by the bulky trimethylammonium group donated by MTSET.

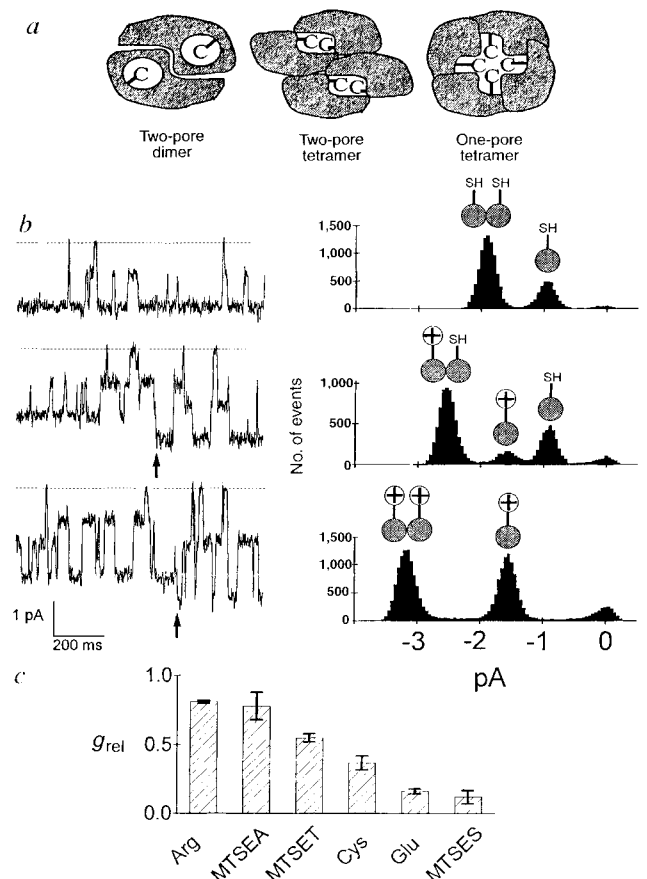


FIG. 4 Two-step reversion of C519 to K519-like channels by MTS reagents. **a**, Illustration of three alternative symmetrical subunit structures of the channel, with C519 drawn to indicate an influential position in the pore. **b**, Response of a C519 channel to  $1 \mu\text{M}$  MTSEA. Channel records at  $-120$  mV (left panel) before MTSEA reaction (upper trace), during the first MTSEA hit (arrow, middle trace), and during the second MTSEA hit (arrow, lower trace). Amplitude histograms (right panel) refer to traces before the first hit, after the first but before the second hit, and after the second hit, respectively; icons associated with histogram peaks represent the modification-state of the C519 side chains in open pores. **c**, Pore conductances,  $g_{\text{rel}}$ , normalized to that of wild-type CIC-0-K519, of C519 modified by MTS reagents and, for comparison, arginine, cysteine and glutamate substitutions.

From these experiments, we deduce two molecular features of the CIC-0 channel. First, the sidedness of MTS modification demonstrates that residue 519 faces the internal solution. The electrostatic influence of this residue suggests that it provides mechanistically relevant surface charge near the cytoplasmic entryway of the Cl<sup>-</sup> conduction pathway. Second, these results provide strong evidence that the CIC-0 channel is a two-pore homodimer, as previously proposed on the basis of velocity-sedimentation behaviour in nondenaturing detergent<sup>10</sup>. Residue 519 exerts its graded electrostatic influence on permeation exactly twice in the channel complex, once separately in each conduction pathway. Moreover, the frequencies of the hybrid C519–K519 and homomeric C519 channels in our preparations are close to the 2 : 1 prediction for a random mixture of equally expressed subunits in a dimer.

The CIC-0 channel is thus constructed by a 2-pore–2-polypeptide architecture unprecedented among eukaryotic ion channels. This structural design is distinct from that of the familiar channels gated by voltage, cyclic nucleotides, or neurotransmitters, which are built around single pores formed on symmetry axes at the junction of 4 or 5 similar subunits<sup>22</sup>. In CIC-0, the twofold axis, assumed to exist in the homodimer, cannot be coincident with either pore. Instead (Fig. 4a), the two identical pores must be formed off-axis<sup>10</sup>, as in the 3-pore–3-polypeptide construction of bacterial porins<sup>23,24</sup>. Consequently, we expect that many parts of the polypeptide contribute pore-determinants, in contrast to the localized pore-forming sequences of voltage-gated cation-conducting channels<sup>25</sup>. CIC-0 is the only member of the CIC family studied at the single-channel level<sup>14</sup>, and so it is not known whether its double-barrelled structure is a unique feature of this homologue or a general property of the entire family. Because we view a membrane protein's quaternary architecture as a fundamental characteristic rather than a modulatable molecular embellishment, we speculate that the two-pore homodimeric character of CIC-0 applies to all members of this large family of Cl<sup>-</sup> channels.

*Note added in proof:* Similar results were obtained by Ludewig *et al.* (*Nature*, this issue pp. 340–343). □

## Methods

**Purification, expression and reconstitution of CIC-0 channels.** Functional CIC-0 channels were purified from *Torpedo californica* electroplax plasma membranes or from HEK-293 cells transiently transfected with a cDNA coding for CIC-0 from *Torpedo marmorata*<sup>1</sup>. CHAPS-solubilized channels were bound to an immunoaffinity resin based on the RM2 epitope (EGQREGLEAVKVTEDP, near residue 650), and eluted with the RM2 peptide as described<sup>10</sup>. Purified CIC-0 (5–10 µg) was reconstituted by gel filtration into proteoliposomes (2 mg ml<sup>-1</sup>), which were subsequently fused into planar lipid bilayers, to give single-channel behaviour identical to that seen without CHAPS treatment<sup>10</sup>. The CIC-0 clone<sup>1</sup> (generously supplied by T. J. Jentsch) was altered by removal of the flanking untranslated sequences and by incorporation of convenient restriction sites, and transferred to the mammalian expression vector pCDNA3 (Invitrogen). HEK-293 cells (50–100-mm plates) were transfected overnight with 0.5 mg CaP<sub>1</sub>-precipitated DNA, collected after 40 h, and extracted with CHAPS for subsequent purification of CIC-0. Hybrid channels were purified similarly after transfection of HEK-293 cells with equal amounts of CIC-0-C519 and of CIC-0-K519 cDNA in which the RM2 epitope had been replaced by the HA epitope.

Single CIC-0 channels were recorded under asymmetrical ionic conditions, with 5 mM Tris-Cl, pH 7.4, and 600 mM NaCl (internal), 200 mM NaCl (external). Records were filtered at 200 Hz and analysed as described<sup>13</sup>. We confined our analysis to properly oriented single channels displaying stationary gating among well defined conductance levels for enough time to collect data at a minimum of 5 voltages; these data-selection criteria applied to over 50% of all the single-channel incorporation events we observed.

**Modification of single channels by MTS reagents.** MTS derivatives<sup>21</sup> carried the following functional groups: ethylamino (MTSEA), ethyltrimethylamino (MTSET), ethylsulphonate (MTSES). After incorporation of a single C519 channel in a bilayer, a control *I*–*V* curve using at least 10 voltages was recorded. The desired methanethiosulphonate reagent (1–10 µM) was then added to the internal solution, with a 15-s burst of vigorous stirring, and data were continuously collected over the next few minutes at a constant holding voltage in the range –80 to –120 mV. Individual 'hits' by MTS reagents were observed as abrupt changes in single-channel current, as in similar experiments on diaphtheria toxin channels<sup>26</sup>.

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- Jentsch, T. J., Steinmeyer, K. & Schwarz, G. *Nature* **348**, 510–514 (1990).
- Pusch, M. & Jentsch, T. *Physiol. Rev.* **74**, 813–825 (1994).
- Jentsch, T. J., Gunther, W., Pusch, M. & Schwappach, B. *J. Physiol. (Lond.)* **482**, 19S–25S (1995).
- Palade, P. T. & Barchi, R. L. *J. Gen. Physiol.* **69**, 879–896 (1977).
- Steinmeyer, K. *et al. Nature* **354**, 304–308 (1991).
- George, A. L., Carackower, M. A., Abdalla, J. A., Hudson, A. J. & Ebers, G. C. *Nature Genet.* **3**, 305–310 (1993).
- Ackerman, M. J. & Clapham, D. E. *Trends Cardiovasc. Med.* **3**, 23–28 (1993).
- Mayer, M. L. *J. Physiol. (Lond.)* **364**, 217–239 (1985).
- Lloyd, S. E. *et al. Nature* **379**, 445–449 (1996).
- Middleton, R. E., Pheasant, D. J. & Miller, C. *Biochemistry* **33**, 13189–13198 (1994).
- Steinmeyer, K., Lorenz, C., Pusch, M., Koch, M. C. & Jentsch, T. J. *EMBO J.* **13**, 737–743 (1994).
- Miller, C. *Phil. Trans. R. Soc. Lond. B* **299**, 401–411 (1982).
- Hanke, W. & Miller, C. *J. Gen. Physiol.* **82**, 25–45 (1983).
- Miller, C. & Richard, E. A. in *Chloride Transporters* (eds Leefmans, A. & Russell, J.) 383–405 (Plenum, New York, 1990).
- Miller, C. & White, M. M. *Proc. Natl Acad. Sci. USA* **81**, 2772–2775 (1984).
- Bauer, C. K., Steinmeyer, K., Schwarz, J. R. & Jentsch, T. J. *Proc. Natl Acad. Sci. USA* **88**, 11052–11056 (1991).
- Pusch, M., Ludewig, U., Rehfeldt, A. & Jentsch, T. J. *Nature* **373**, 527–531 (1995).
- Cooper, E., Couturier, S. & Ballivet, M. *Nature* **350**, 235–238 (1991).
- Liu, D. T., Tibbs, G. R. & Siegelbaum, S. A. *Neuron* **16**, 983–990 (1996).
- Akabas, M. H., Stauffer, D. A., Xu, M. & Karlin, A. *Science* **258**, 307–310 (1992).
- Stauffer, D. A. & Karlin, A. *Biochemistry* **33**, 6840–6849 (1994).
- Hille, B. *Ionic Channels of Excitable Membranes* (Sinauer, Sunderland, MA, 1992).
- Weiss, M. S. & Schulz, G. E. *J. Mol. Biol.* **227**, 493–509 (1992).
- Cowan, S. W. *et al. Nature* **358**, 727–733 (1992).
- MacKinnon, R. *Neuron* **14**, 889–892 (1995).
- Mindell, J. A., Zhan, H., Huynh, P. D., Collier, R. J. & Finkelstein, A. *Proc. Natl Acad. Sci. USA* **91**, 5272–5276 (1994).

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## Two physically distinct pores in the dimeric CIC-0 chloride channel

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**THE *Torpedo* chloride channel CIC-0 (ref. 1) is the prototype of a large family of chloride channels that have roles in transepithelial transport<sup>2</sup> and in regulating electrical excitability<sup>3–6</sup> and cell volume<sup>7</sup>. CIC-0 opens in bursts with two identical conductance levels of ~8 pS (refs 8–10). Hyperpolarization slowly increases the probability of bursts ('slow gating'), and depolarization increases channel opening within bursts ('fast gating'). Replacing serine 123 by threonine changes rectification, ion selectivity and gating, but retains the typical bursting behaviour with two identical independent albeit reduced, conductance states (~1.5 pS). Coexpression with wild-type CIC-0, either as covalently linked concatamers or as independent proteins, leads to bursting channels with two different pores. Our experiments strongly suggest that conductance, ion selectivity and 'fast' gating are determined only by the single subunit forming a single pore, independent from the attached pore; in contrast, 'slow' gating is a function of both subunits. Thus CIC-0 is a homodimer with two largely independent pores.**

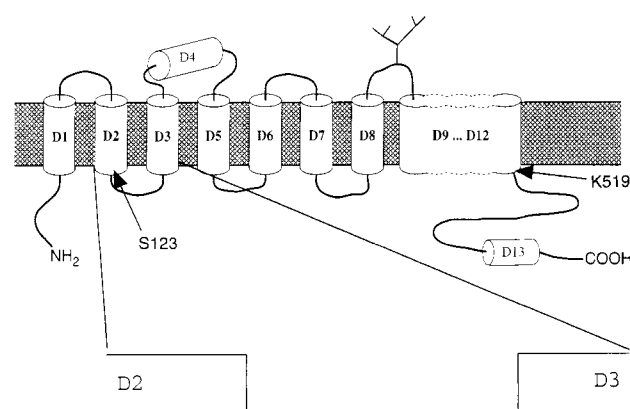
A region between transmembrane domains D2 and D3 is highly conserved across all known CIC proteins<sup>11</sup>, including those from yeast<sup>12</sup> and bacteria<sup>13</sup> (Fig. 1). In CIC-0, a conservative substitution of serine 123 by threonine (S123T) led to fast gating being slowed (Fig. 2a) and a slight outward rectification of open channels and a relative loss of ion selectivity (Fig. 2b). Wild-type CIC-0 activates slowly at negative voltages owing to the voltage-dependent slow gate, but no such effect was observed with S123T up to –150 mV (Fig. 2c).

The dominant negative effects of CIC-1 mutations found in myotonia congenita indicate that CIC-1 is a homomultimeric channel<sup>15,6</sup>. We therefore investigated whether wild-type (WT) and S123T CIC-0 proteins can form heteromultimeric channels, and constructed head-to-tail concatameric channels (S123T-WT and WT-S123T). Expression of control WT-WT concatamers (Fig. 2) yielded currents indistinguishable from wild-type CIC-0. Macroscopic current measurements indicate that rectification, ion selectivity and fast gating of the S123T-WT concatamer have properties very similar to those of the wild type (Fig. 2a, b). However, there was no slow activation by hyperpolarization up to -150 mV (Fig. 2c). Similar results were obtained with a concatamer of reverse order (WT-S123T). These results cannot be explained by a superposition of independent wild-type and S123T currents, and indicate the formation of heteromultimeric channels.

Similar to the wild type, S123T channels show two identical subconductance levels (Figs 3c and 4a) within bursts. In contrast to the ~8 pS conductance of wild-type CIC-0 and WT-WT concatamers (Fig. 3a), S123T single-channel conductance was only ~1.5 pS. Consistent with the slower macroscopic current relaxations (Fig. 2a), single-channel gating time constants were increased by about threefold.

S123T-WT concatamers show ~1.5, ~8 and ~9.5 pS conductance levels (Figs 3b, d and 4a). Similar results were obtained for WT-S123T concatamers (Fig. 3e) and with coexpression of non-linked S123T and wild-type CIC-0 proteins (data not shown). As expected, we also observed homomeric wild-type or S123T channels in the latter case. Importantly, the heteromultimer also displays long time stretches of missing channel activity (Fig. 3d). Hence these channels (and S123T homomers) retain a slow gate operating on all three conductance levels, although it has lost its voltage dependence.

On the basis of single-channel analysis of the native *Torpedo* channel, and on effects of the inhibitor 4,4'-diisothiocyanato-stilbene-2,2'-disulphonic acid (DIDS), a 'double barrelled' channel with two identical pores was proposed<sup>8,9</sup>. In this model, WT-S123T channels would have two different pores, one WT pore of ~8 pS and one mutant pore of ~1.5 pS. We show that this model is correct, and that the permeation and gating properties of an individual pore are independent of the second attached pore. Replacement of intracellular chloride by bromide reduced the conductance of the large pore of WT-S123T channels by ~50%, although the less selective small pore was reduced by only ~20% (Fig. 3e, f). This reduction is independent of the state (open or

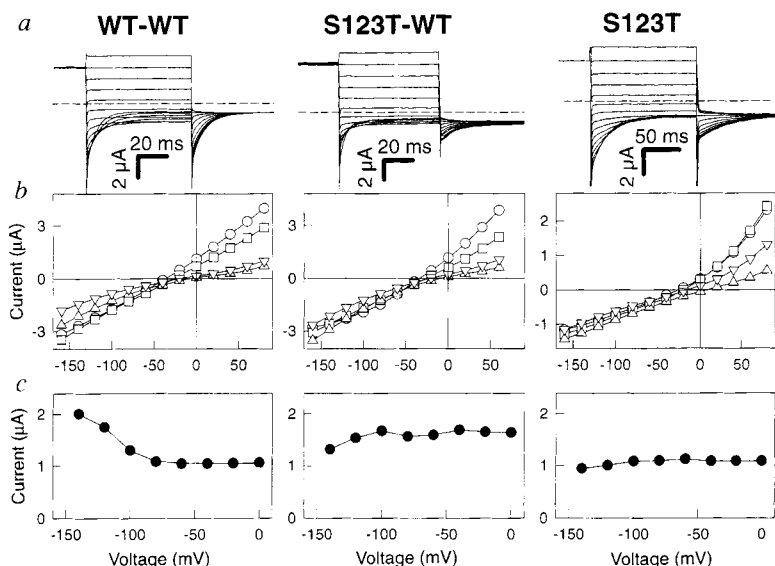


| mutant | 123                                        |
|--------|--------------------------------------------|
| CIC-0  | IVSPQAVGSGIPELKTIIRGAVL...HEYLTIRTFVAKTVGL |
| hC1C-1 | LISPPAVGSGIPEMKTILRGVVL...KEYLTMKAFVAKVVAL |
| hC1C-5 | VFAPVACGSGIPEIKTILSGFII...RGYLGKWTLVIKTITL |
| hC1C-7 | FIEPVAAGSGIPQIKCFLNGVKI...PHVRLKTLVIKVSQV  |
| scC1C  | YVAPMATGSGISEIKVWVSGFEYNKEFLGLTLVIKVSVAL   |
| ecC1C  | KYAPMATGSGIPEIEGALED...QRPVRWVRVLPVKFFGG   |

FIG. 1 Topology model<sup>14</sup> of the CIC-0 chloride channel and location of mutations. S123 is located in a highly conserved stretch at the proposed cytoplasmic end of transmembrane domain D2, and K519 is located at the end of D12. Strongly divergent CIC members are given for comparison: hC1C-1, human muscle Cl<sup>-</sup> channel mutated in myotonia<sup>4-6</sup>; hC1C-5, human kidney Cl<sup>-</sup> channel mutated in kidney-stone diseases<sup>2</sup>; hC1C-7, a broadly expressed human CIC member<sup>11</sup>; scC1C, *Saccharomyces cerevisiae* CIC<sup>12</sup>; and ecC1C, a CIC from *Escherichia coli*<sup>13</sup>. Bold type indicates conserved sequences.

closed) of the second pore, and does not depend on the identity of the attached pore, as similar ion selectivities are found for homomeric wild-type or S123T channels, respectively (data not shown). The same independence holds for open probabilities of both pores (Fig. 4b, c). Further, gating kinetics of the 8-pS pore are independent of whether it is associated with a wild-type or mutant pore, and the (difficult to measure) mutant S123T pore roughly retained its slower gating kinetics within the heteromer (data not shown). Slow gating, in contrast, which acts on both pores simultaneously, depends on both pores, as the apparent voltage independence of slow gating of the S123T mutant was imposed on the heteromeric S123T-WT channel (Fig. 2c).

FIG. 2 Macroscopic currents of CIC-0 mutants and concatamers as determined by two-electrode voltage clamping. Results are shown for WT-WT concatamers (left), S123T-WT concatamers (middle) and S123T CIC-0 mutant monomers (right). WT-WT concatamers yield currents indistinguishable from wild-type channels<sup>10,14</sup>, and WT-S123T concatamers yield currents similar to S123T-WT concatamers (data not shown). a, Typical traces at 104 mM chloride (ND96). After activating the slow gate by hyperpolarization and a prepulse to +60 mV to open maximally the fast gate, oocytes were clamped sequentially to voltages between +80 and -160 mV for 75 ms (WT-WT and S123T-WT) or 150 ms (S123T), followed by a constant pulse to -100 mV. Hyperpolarization closes the fast gate. The broken line indicates zero current. b, Instantaneous (open pore) currents measured at the beginning of the test pulse with partial replacement of external chloride (circles) by bromide (squares), iodide (triangles), and nitrate (inverted triangles). Note the slight outward rectification and relative loss of ion selectivity with the S123T mutant. c, Currents at +60 mV as a function of hyperpolarizing prepulses of 5 s duration. This voltage activation (by the 'slow gate') is lost both in the S123T mutant and in the S123T-WT concatamer. Because wild-type conductance is about fivefold larger than S123T conductance (Fig. 3), macroscopic currents of heteromers resemble wild-type currents except for the absence of slow gating.





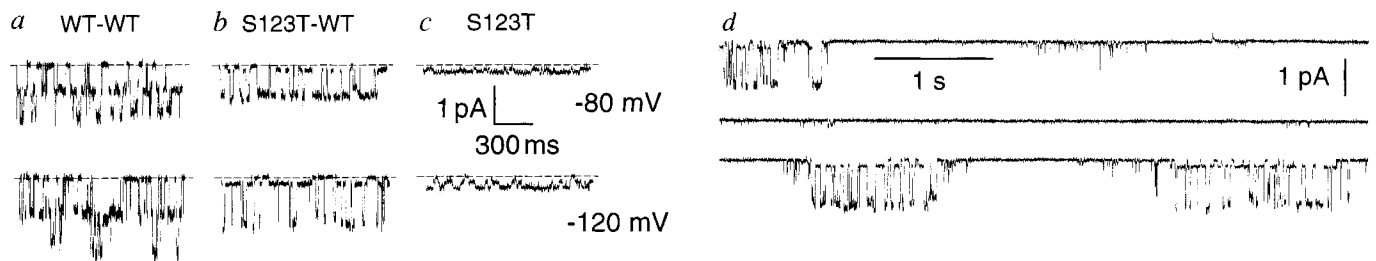
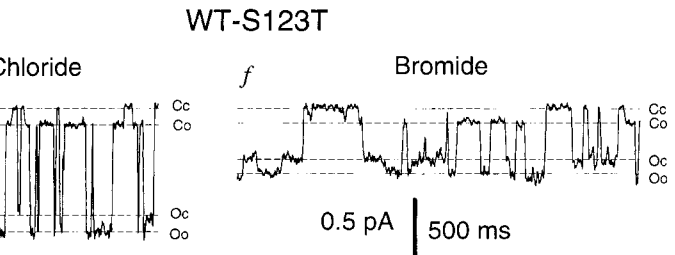


FIG. 3 Single-channel analysis of WT-WT (a), S123T-WT concatamers (b) and the S123T mutant (c) at different holding potentials. Note the two equal conductance levels ( $\sim 8$  pS) with WT-WT concatamers and with S123T ( $\sim 1.5$  pS), and the superposition of one large ( $\sim 8$  pS) and one small ( $\sim 1.5$  pS) level with the S123T-WT concatamer. Open probability increases with depolarization in all cases. The broken line indicates the fully closed state. d, Longer continuous recording (at  $-120$  mV) of a S123T-WT channel showing that the slow gate closes both conductance levels (pores). e, Recording of a WT-S123T channel with high cytoplasmic chloride concentration (160 mM). f, The same patch after replacement of chloride by bromide; the conductance of the large (wild-type) pore is reduced by 50%, and the



mutant pore by only 20%. Similar effects were seen in 5 patches. Nomenclature (Cc, Co, Oc, Oo) is explained in the Fig. 4 legend.

To exclude the possibility that these observations are specific for the S123T mutant, we studied a mutant<sup>14</sup> (K519E) at a very different position (Fig. 1). It yielded a bursting channel with two superimposed conductance levels of  $\sim 1$  pS (Fig. 5a). With a WT-K519E concatamer, we again observed three conductance levels ( $\sim 1$ ,  $\sim 8$  and  $\sim 9$  pS) (Fig. 5b), suggesting the presence of independent  $\sim 8$ -pS and  $\sim 1$ -pS pores.

Our experiments indicate a simple dimeric structure of ClC-0 (Fig. 6A). With alternative tetramer models, where each pore is formed by two subunits (Fig. 6C-F), one would expect to find (WT/WT), (mutant/mutant), and (mutant/WT) pores with different conductances. However, when co-injecting monomeric mutant S123T and wild-type subunits, we only observed 8/8 pS, 1.5/1.5 pS, and 8/1.5 pS channels (8 patches), never double-barrelled channels with other conductances. With S123T-WT and WT-S123T concatamers (injected singly), we again only observed 8/1.5 pS channels (14 patches). In principle, S123T subunits may associate preferentially with themselves, but this seems unlikely as WT-K519E concatamers again had one large and one small pore ( $n = 3$ ). Another explanation could be that this linkage forces the subunits to assemble into mutant-mutant and WT-WT pores

(Fig. 6D, F). We therefore co-injected oocytes with S123T-WT and WT-S123T concatamers at a 1:1 ratio. One now would expect to find 8/1.5 pS channels in half of the cases (as a result of WT-S123/WT-S123 (Fig. 6D) and S123T-WT/S123-WT complexes (Fig. 6F), and S123T-WT/WT-S123 channels in the remaining 50% (Fig. 6E). These latter channels should have two identical conductance levels of unknown (but probably intermediate) magnitude. We only observed 8/1.5 pS channels in all 12 patches displaying 'double-barrelled' channels. Assuming equal association efficiencies, this reduces the probability of the tetramer model to 0.03%. Thus, consistent with a sedimentation analysis of ClC-0 protein<sup>15</sup>, our experiments strongly favour a homodimeric structure of ClC-0. However, analysis of dominant-negative mutants suggested that ClC-1 has more than two subunits<sup>5</sup>. It seems impossible that the number of subunits lining a pore differs between these channels, but the interactions between individual pores may be different. Indeed, several dominant-negative mutations were found<sup>5,6</sup> in ClC-1, but we have not yet been able to construct dominant-negative mutants in ClC-0.

In a homodimer, either each single subunit forms one pore by

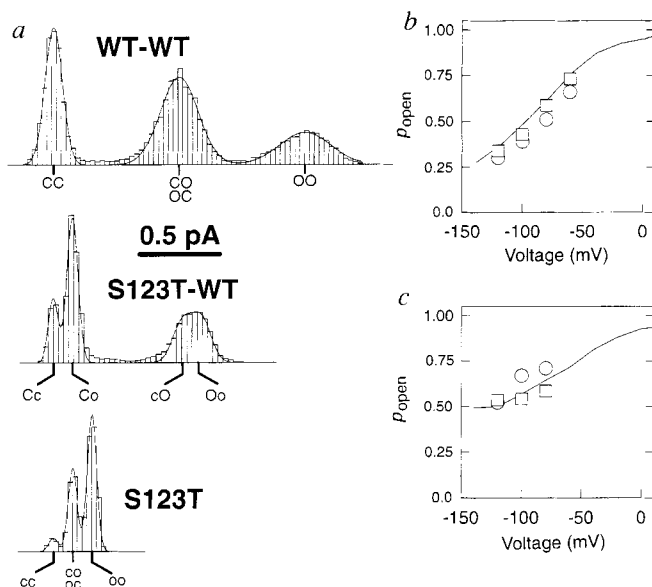


FIG. 4 Analysis of single-channel data. a, Amplitude histograms of single-channel recordings (within bursts, that is, with open 'slow gate') of WT-WT and S123T-WT concatamers, and the S123T mutant at  $-100$  mV. With WT-WT concatamers (and with wild-type ClC-0 (ref. 10)), and with S123T mutants, three distinct, equidistant conductance levels are seen, which are due to two closed pores (CC), one closed and one open pore (CO or OC, which are indistinguishable because both pores have equal conductance), and two open pores (OO). The two pores of the S123T-WT concatamer have different conductances, yielding different currents for Co and Oc (lower-case letters refer to the smaller S123T pore), giving four current levels. The transitions between Oc and Oo levels cannot be clearly resolved owing to the low conductance of the S123T pore and the noise from the open wild-type pore. Lines indicate fits of independent gaussian distributions, with four distributions fitted for the heteromers. Marks indicate maxima of these fits. Conductances are nearly unaffected by the second pore: for the wild-type (which can be resolved quite clearly) conductance was  $7.8 \pm 0.4$  pS ( $n = 3$ ) in WT-WT dimers, and  $8.6 \pm 0.4$  pS ( $n = 6$ ) in S123T-WT dimers. b, c, Comparison of open probability ( $p_{\text{open}}$ ) of the fast gate from macroscopic measurements (line) with those derived from single-channel analysis (data points) for WT (b) and S123T (c) pores. Results are for pores within homomeric channels (WT-WT concatamers or S123T channels) (circles) and pores within the heteromeric S123T-WT channel (squares).

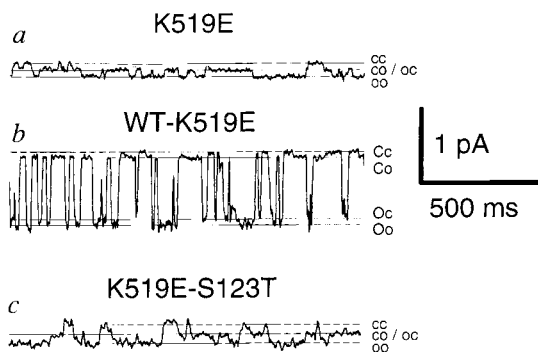


FIG. 5 Analysis of the K519E mutant, and of the WT-K519E and K519E-S123T concatamers. *a*, A single K519E channel held at  $-120$  mV has two  $\sim 1$  pS pores; WT-K519E concatamers (*b*) yield channels with one  $\sim 8$  pS (wild-type) pore and one  $\sim 1$  pS mutant pore. The  $\sim 1$  pS conductance is consistent with previous noise analysis<sup>14</sup>. *c*, The K519E-S123T tandem channel yields two small ( $\sim 1$ – $1.5$  pS) pores. Macroscopic currents were strongly outwardly rectifying (data not shown), as expected for S123T and K519E pores. (A linear wild-type pore with its larger conductance would dominate the current (see Fig. 2b for the S123T-WT concatamer).) This supports the model in Fig. 6Aa, Ba. Lettering as in Fig. 4.

itself (Fig. 6Aa), or both CIC-0 monomers contribute (with different portions) to each of the two pores (Fig. 6Ab). A K519E-S123T dimer should discriminate between these possibilities (Fig. 6B). Single-channel analysis revealed two small pores (Fig. 5c), strongly suggesting (but not proving) that a single subunit forms a single pore.

Our experiments have also identified a new region determining pore properties and fast gating which, at the primary sequence level, is remote from the end of D12 that also affects these properties<sup>14</sup>. As one pore is formed by a single subunit (or, less likely, by two different parts of the two subunits), one would indeed expect that several regions of the protein participate in pore formation. This is in contrast to the situation found in cation channels<sup>16</sup>.

*Note added in proof:* Similar results were obtained by Middleton *et al.* (*Nature*, this issue pp. 337–340). □

## Methods

**Functional expression in *Xenopus* oocytes.** Mutant and concatameric CIC-0 cDNAs were constructed by recombinant PCR. Construction of concatamers introduced four residues (GTTS) between the monomers. Capped cRNA was transcribed *in vitro* from constructs in the vector pTLN<sup>6</sup>, 5–10 ng of which was injected into *Xenopus* oocytes<sup>1</sup>. Currents were measured after 2–4 days by two-electrode voltage-clamping or patch-clamping at room temperature. Standard saline was ND96 (ref. 5) and contained 104 mM Cl<sup>−</sup>. For Fig. 2b, 80 mM Cl<sup>−</sup> was replaced by equivalent amounts of other anions. Single-channel recordings were performed in the inside-out configuration of the patch-clamp technique. The external solution contained (in mM): 95 N-methyl-D-glucamine-Cl (NMDG-Cl), 5 MgCl<sub>2</sub> and 5 HEPES, pH 7.3. The internal solution contained 95 NMDG-Cl (or 160 NMDG-Cl or NMDG-Br for Fig. 3e, f and Fig. 5), 2 MgCl<sub>2</sub>, 5 EGTA and 5 HEPES, pH 7.3. Currents were filtered at 300 Hz (S123T and K519E) and 500 Hz (other constructs).

**Data analysis.** Amplitude histograms were constructed from single-channel traces of 7 s duration using the Biopatch program (Biologic, Grenoble). For kinetic analysis, idealized current traces (>300 events) were constructed with a 50% threshold criterion, followed by visual inspection of each transition. From this, mean dwell times were determined ( $\tau_{oc}$ ,  $\tau_{oc}$ , and  $\tau_{oo}$  for homomeric channels;  $\tau_{oc}$ ,  $\tau_{oc}$ ,  $\tau_{oo}$  and  $\tau_{oo}$  for heteromeric channels). For homomeric channels, opening rate  $\alpha$ , and closing rate  $\beta$  were calculated as  $\alpha = 1/(2\tau_{oc})$ , and  $\beta = 1/(2\tau_{oo})$ . See Fig. 4 legend for nomenclature. Because open wild-type pores have an increased noise level, openings of the small mutant pore on top of open wild-type pores could not be analysed quantitatively (that is,  $\tau_{oc}$  and  $\tau_{oo}$  were unreliable). Gating rates could, however, be obtained using the following procedure. First, we determined the well-defined dwell times of the lumped states  $\tau_{oc+oc}$  and  $\tau_{oo+oo}$  (ignoring transitions of the small mutant pore) from which we calculated  $\alpha_{WT} = 1/\tau_{oc+oc}$ ,  $\beta_{WT} = 1/\tau_{oo+oo}$ . From these values we obtained  $\alpha_{mutant}$  and  $\beta_{mutant}$  from  $\tau_{oc}$  and  $\tau_{oo}$  assuming that  $\tau_{oc} = 1/(\alpha_{WT} + \alpha_{mutant})$ , and

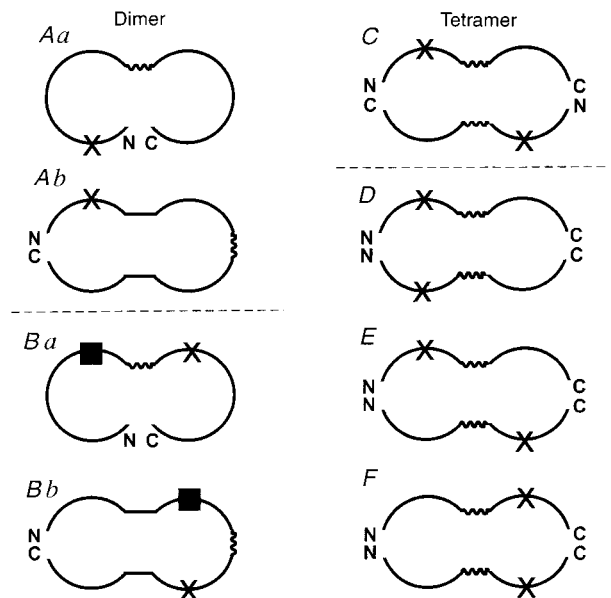


FIG. 6 Possible models for the multimeric structure of CIC-0 based on experiments using various concatamers. The most simple models assumes a dimer, with either one pore per monomer (Aa) or a pore formed together by different portions of both subunits (Ab). A concatamer of two CIC-0 monomers connected by a linker (wavy line) is depicted. Amino (N) and carboxy (C) termini are shown. In both models, a single mutation (indicated by X) will affect only one pore. The models in A can be differentiated when mutations are inserted in the second part of the first subunit and the first part of the second one (panels B) (for example: X, S123T; square, K519E). Ba predicts two mutant pores; Bb predicts one wild-type and one mutant pore. Our experiments (Fig. 5c) support model Aa, Ba, C–F. Hypothetical tetramers, in which each pore is formed by two CIC monomers. The symmetrical (and more feasible) model in C predicts channels with two identical 'barrels'; this seems to be excluded, as we always observed two different 'barrels' with S123T-WT, WT-S123T and WT-K519E concatamers. The alternative model (D–F) is *a priori* much less likely, as it violates symmetry (as shown, different 'sides' of the protein would face the pore). However, the models in D and F both predict channels with two different barrels, as observed experimentally. Assuming equal association efficiencies, half of the channels produced by coexpressing both concatamers should assemble as in E. These will have two equal (heteromeric) barrels, which we never observed. Thus our experiments strongly favour the simple dimeric model shown in Aa, Ba.

$\tau_{oc} = 1/(\alpha_{WT} + \beta_{mutant})$ . The probability of being open ( $p_{open}$ ) was calculated as  $p_{open} = \alpha/(\alpha + \beta)$ . This measure of  $p_{open}$  was indistinguishable from values obtained from the gaussian fits to the amplitude histograms.

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- Jentsch, T. J., Steinmeyer, K. & Schwarz, G. *Nature* **348**, 510–514 (1990).
- Lloyd, S. E. *et al.* *Nature* **379**, 445–449 (1996).
- Steinmeyer, K. *et al.* *Nature* **354**, 304–308 (1991).
- Koch, M. C. *et al.* *Science* **257**, 797–800 (1992).
- Steinmeyer, K., Lorenz, C., Pusch, M., Koch, M. C. & Jentsch, T. J. *EMBO J.* **13**, 737–743 (1994).
- Pusch, M., Steinmeyer, K., Koch, M. C. & Jentsch, T. J. *Neuron* **15**, 1455–1463 (1995).
- Gründer, S., Thiemann, A., Pusch, M. & Jentsch, T. J. *Nature* **360**, 759–762 (1992).
- Miller, C. *Phil. Trans. R. Soc. Lond. B* **299**, 401–411 (1982).
- Miller, C. & White, M. M. *Proc. Natl Acad. Sci. USA* **81**, 2772–2775 (1984).
- Bauer, C. K., Steinmeyer, K., Schwarz, J. R. & Jentsch, T. J. *Proc. Natl Acad. Sci. USA* **88**, 11052–11056 (1991).
- Brandt, S. & Jentsch, T. J. *FEBS Lett.* **377**, 15–20 (1995).
- Greene, J. R., Brown, N. H., DiDomenico, B. J., Kaplan, J. & Eide, D. J. *Mol. Gen. Genet.* **241**, 542–553 (1993).
- Fujita, N., Mori, H., Yura, T. & Ishihama, A. *Nucleic Acids Res.* **22**, 1637–1639 (1994).
- Pusch, M., Ludwig, U., Rehfeldt, A. & Jentsch, T. J. *Nature* **373**, 527–531 (1995).
- Middleton, R. E., Pheasant, D. J. & Miller, C. *Biochemistry* **33**, 13189–13198 (1994).
- Jan, L. Y. & Jan, Y. N. *Nature* **371**, 119–122 (1994).

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# Cooperation of Stat2 and p300/CBP in signalling induced by interferon- $\alpha$

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THE transcription factor ISGF3 transduces interferon (IFN)- $\alpha$  signals and activates the transcription of cellular antiviral defence genes<sup>1,2</sup>. Adenovirus E1A blocks the IFN- $\alpha$  response, allowing unhindered viral replication<sup>3-5</sup>. ISGF3 consists of Stat1, Stat2 and p48. Here we show that p300 and/or CBP (CREB-binding protein), which are transcription adaptors targeted by E1A, interact specifically with Stat2. Binding occurs between the first cysteine-histidine-rich region of p300/CBP and the carboxy-terminal segment of Stat2, a domain essential for ISGF3 function<sup>6</sup>. We find that this domain of Stat2 has transactivation potential, which correlates with its binding to p300/CBP. Moreover, E1A represses Stat2 transactivation and IFN- $\alpha$ -activated transcription by inhibiting p300/CBP function. This provides a new mechanism for inhibition of the IFN- $\alpha$ -activated antiviral response by E1A, and supports the view that E1A binding to p300/CBP has functional significance for adenovirus replication in its natural host.

p300 and CBP are ubiquitous, evolutionarily conserved, nuclear phosphoproteins that function, at least in part, by linking several different signal-responsive transcriptional activators to the basal transcription apparatus<sup>7-11</sup>. p300 and CBP contain three cysteine-histidine-rich segments, referred to as CH1, CH2 and CH3 (Fig. 1a). Adenoviral E1A binds to CH3 through the E1A amino terminus and CR1 domains, and represses p300 transcriptional activity<sup>12,13</sup>. In a screen for proteins that interact with the CH1 segment of p300/CBP, we isolated two overlapping Stat2 clones (Fig. 1a). *In vitro* translated Stat2 bound specifically to immobilized GST-p300 (GST-CH1), and this binding required the C-terminal 83 amino acids of Stat2 (Fig. 1b). Anti-p300/CBP immunoprecipitates from extracts of IFN- $\alpha$ -stimulated cells contained Stat2 (Fig. 2a). In addition, anti-Stat2 immunoprecipitates from extracts of IFN- $\alpha$ -stimulated cells contained p300/CBP (Fig. 2b). These results indicate the presence of specific complexes *in vivo*, that contain both p300/CBP and Stat2.

The binding of IFN- $\alpha$  to its receptor results in the formation and nuclear translocation of the Stat2-containing transcription factor, ISGF3 (refs 1, 6, 14). ISGF3 binds to IFN- $\alpha$  signal-response elements (ISREs), and activates the transcription of downstream antiviral genes, for example 2'-5'-oligoadenylate synthetase. The C terminus of Stat2 is essential for ISGF3 function<sup>6</sup>, but is dispensable for the formation of the DNA-binding ISGF3 complex. We therefore investigated whether the C terminus of Stat2 could serve as a transcriptional activation domain.

The C-terminal 181 residues of Stat2, fused to the GAL4 DNA-binding domain (GAL4-Stat2), led to transactivation of a GAL4-luciferase reporter (Fig. 3a). Deletion of the C-terminal 83 amino acids of Stat2 (GAL4-Stat2 $\Delta$ ) resulted in a marked reduction in transcriptional activity (Fig. 3a). The GAL4-Stat2 and GAL4-Stat2 $\Delta$  fusion proteins were expressed at comparable levels (data not shown). Therefore, the C terminus of Stat2 has transactivation potential which correlates closely with its ability to bind p300 *in vitro*. Substitution of the C-terminal 83 residues of Stat2 with the VP16 transactivation domain restored the transcriptional activity of GAL4-Stat2 $\Delta$  (Fig. 3a). Furthermore, co-transfection of p300

(fused to the VP16 transactivation domain) increased GAL4-Stat2 transactivation activity fivefold, when compared to a control transfection with a p300-VP16 fusion lacking the CH1 region (Fig. 3b). Hence, the p300 CH1 domain seems to be necessary for *in vivo* Stat2 binding. Similar results were obtained using the 3  $\times$  GAL4-luciferase reporter in this assay, although it was less effective, probably because of the greater stability of CAT compared with luciferase. The same interaction was also demonstrated by the yeast two-hybrid assay, using lexA-p300 CH1 as bait and Stat2 as prey plasmids (data not shown).

E1A inhibits ISGF3 transactivation and the cellular response to IFN- $\alpha$ , relieving the IFN- $\alpha$ -mediated block to viral replication<sup>3-5</sup>. The observation that Stat2 binds to p300/CBP indicated that the Stat2-containing ISGF3 complex may function in transcriptional activation partly through the action of p300/CBP. Thus a possible mechanism for the effect of E1A on IFN- $\alpha$ -activated ISGF3

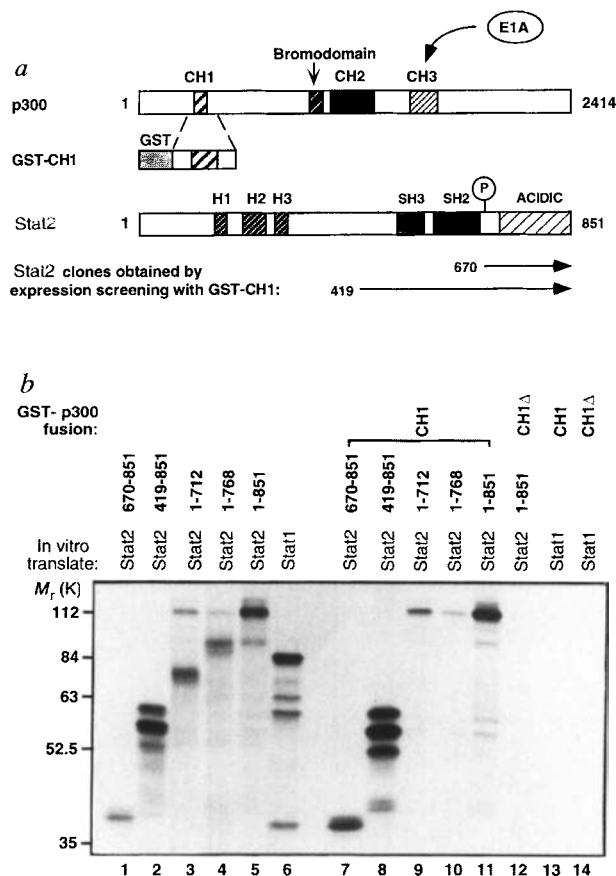


FIG. 1 a, p300 (and CBP) contain 3 cysteine-histidine rich regions (CH1-3). In an expression screen using GST-CH1 as a probe, we isolated two overlapping Stat2 clones, encoding residues 419-851, and 670-851, respectively. Stat2 contains an acidic C-terminal segment which is unique within the STAT family. Stat2 helical domains (H1, H2 and H3), SH3 and SH2 domains, and a specific tyrosine residue that is phosphorylated (P) upon activation<sup>6</sup> are indicated. b, Lanes 1 and 2: *in vitro* translated, <sup>35</sup>S-labelled, truncated Stat2 species containing residues 670-851 ( $M_r$ , 37K) and 419-851 ( $M_r$ , 60K). Lanes 3 and 4: Stat2 residues 1-712 ( $M_r$ , 70K) and 1-768 ( $M_r$ , 90K) respectively. In lanes 3 and 4, a small amount of full-length Stat2 ( $M_r$ , 112K) was noted, due to incomplete enzymatic digestion of the DNA template. Lanes 5 and 6: full-length Stat2 and Stat1 ( $M_r$ , 84K), respectively. Lanes 7-14: the binding of the <sup>35</sup>S-labelled proteins to GST-CH1 or GST-CH1 $\Delta$  (a mutant lacking the CH1 region), immobilized on glutathione-Sepharose beads, was assessed. Note that in lanes 9 and 10, the small amount of full-length Stat2 present in the translate (lanes 3 and 4) bound efficiently, whereas the C-terminally truncated proteins failed to bind. Lanes 1-6 contain 20% of the *in vitro* translated protein present in the reaction mixtures that gave rise to bands in lanes 7-14.

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transactivation is the inhibition of p300/CBP. If true, E1A inhibition of ISGF3 transactivation function should depend upon the binding of E1A to p300/CBP. Using the 2'-5'-oligo(A) synthetase ISRE<sup>16</sup> fused to a luciferase reporter, we investigated whether E1A mutants, defective in p300/CBP binding could repress IFN- $\alpha$ -stimulated transcription. These experiments were performed in U-2 OS human osteosarcoma cells, in which the luciferase reporter was activated sevenfold by IFN- $\alpha$  (data not shown).

Both 12S E1A and the control E1A $\Delta$ 121-150 mutant (which still binds p300/CBP, but not retinoblastoma family proteins<sup>15,16</sup>), led to ~fivefold repression of IFN- $\alpha$ -stimulated luciferase activity when compared with a vector control (Fig. 4a). However, mutants bearing N-terminal ( $\Delta$ 2-36), or CR1 ( $\Delta$ 30-85) deletions, which are defective for p300/CBP binding<sup>15,16</sup>, were only minimally active in this regard (Fig. 4a). These results indicate that the binding of E1A to p300/CBP is responsible for the inhibition of ISGF3

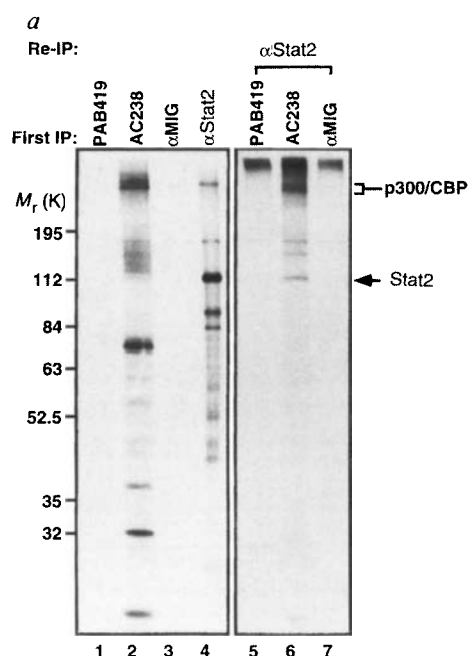
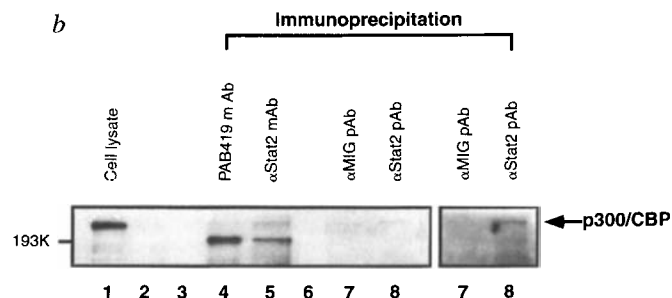


FIG. 2 a, Re-immunoprecipitation with anti-Stat2 of <sup>35</sup>S-methionine labelled p300/CBP immunoprecipitates from IFN- $\alpha$  stimulated HeLa cell extracts. Lanes 1-4: immunoprecipitation with monoclonal antibodies



PAB419 (control), anti-p300/CBP (AC238), affinity-purified rabbit anti-mouse IgG ( $\alpha$ MIG, from Cappel, as control), or affinity-purified polyclonal anti-Stat2 C terminus ( $\alpha$ Stat2, sc-476; Santa Cruz), respectively. Lanes 5-7: labelled proteins obtained by re-immunoprecipitation with  $\alpha$ Stat2. Lane 6 contains a band that comigrated with Stat2. There is also a band corresponding to p300/CBP, probably owing to the presence of renatured AC238 antibody. Lanes 1-4 are shown after 2 days, and lanes 5-7 after 7 days of autoradiography. b, Anti-p300/CBP western blot of anti-Stat2 immunoprecipitates (IP) from IFN- $\alpha$ -stimulated U-2 OS cell extracts. Film exposure time of the blot was 30 min. Lane 1 contains 3  $\mu$ g of U-2 OS cell lysate, to show the position of p300/CBP. Immunoprecipitations were done with monoclonal antibodies PAB419 (as control, lane 4), monoclonal antibody against Stat2 N terminus (S21220, lane 5; from Transduction Labs),  $\alpha$ MIG (lane 7, as control), and affinity-purified polyclonal antibody against the Stat2 N terminus (sc-839, lane 8; Santa Cruz). Lanes 2, 3 and 6 contained size markers (not shown). Side panel: lanes 7 and 8 are shown after a longer exposure time (~1 h). Abbreviations: mAb, monoclonal antibody; pAb, polyclonal antibody.

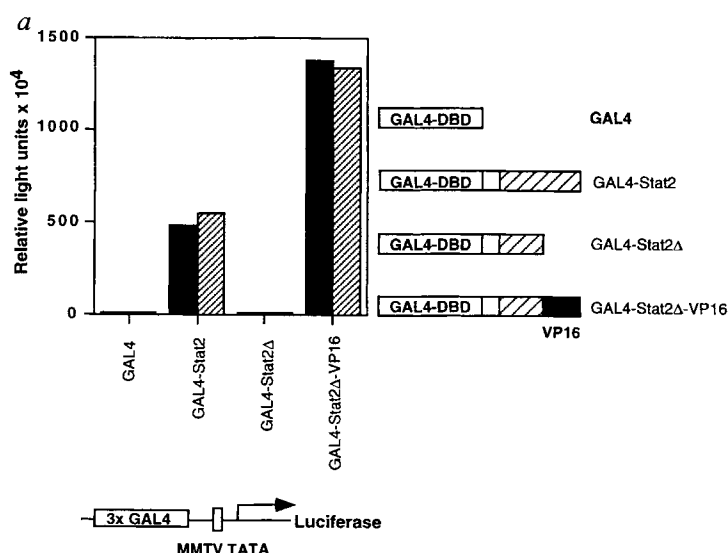
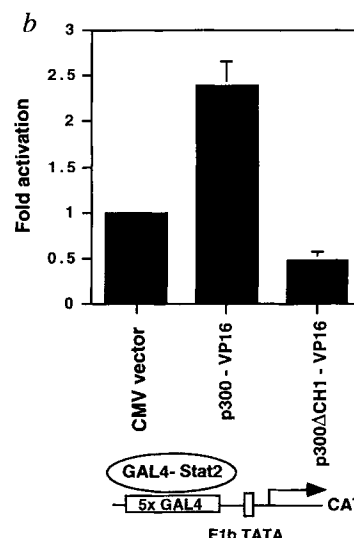


FIG. 3 a, Transcriptional activation of a GAL4-luciferase reporter by co-transfected GAL4, GAL4-Stat2, GAL4-Stat2 $\Delta$  (a mutant deleting the p300/CBP binding domain), and GAL4-Stat2 $\Delta$ -VP16, a mutant that carries the VP16 transactivation domain in place of that of Stat2. The results of a representative experiment are shown in which duplicate transfection mixtures (filled and hatched bars) were tested. b, Fold activa-



tion (mean + standard deviation) of GAL4-Stat2 activation of a GAL4-CAT reporter, by co-transfected CMV-p300-VP16, or CMV-p300 $\Delta$ CH1-VP16, compared with the effect of a backbone CMV vector (set at 1). The results of two independent experiments are shown. The second experiment was done in duplicate.



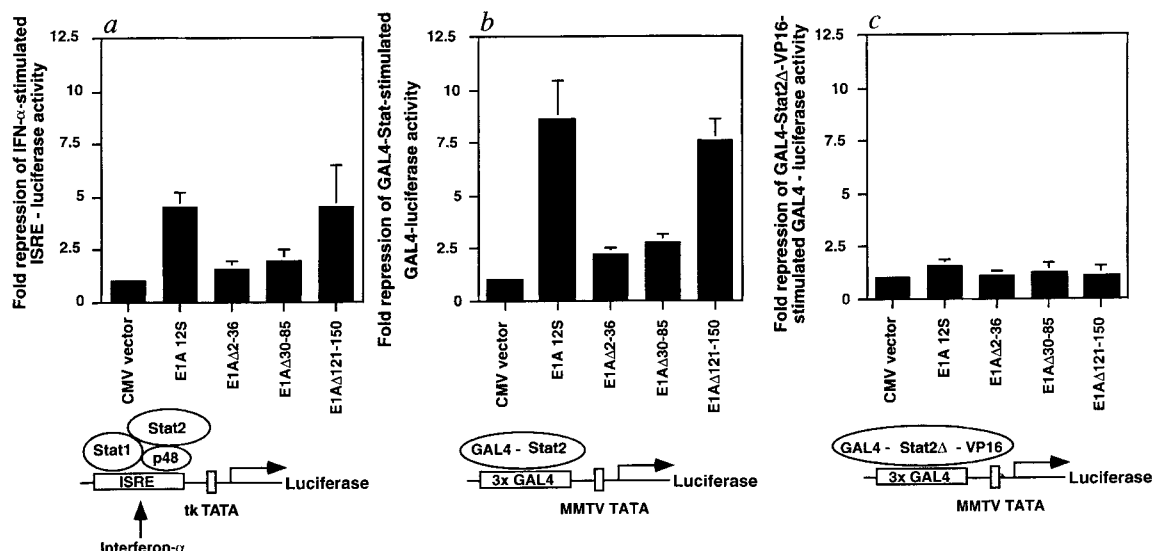


FIG. 4 a, Fold repression of IFN- $\alpha$ -stimulated transactivation of an ISRE-luciferase reporter in U-2 OS cells, by co-transfected wild-type E1A 12S, E1A $\Delta$ 2-36, E1A $\Delta$ 30-85, and E1A $\Delta$ 121-150. b, Fold repression of GAL4-Stat2 transactivation of a GAL4-luciferase reporter in U-2 OS cells, by co-transfected wild-type E1A or mutant plasmids. c, Fold repression of GAL4-Stat2 $\Delta$ -VP16 transactivation of a GAL4-luciferase reporter, in U-2 OS cells, by co-transfected wild-type E1A or mutant plasmids. In each

panel, luciferase activity, normalized for LacZ activity (that is, transfection efficiency), was used to calculate fold repression with respect to an otherwise identical control transfection with CMV backbone vector, which was set at 1. Results are presented as the mean  $\pm$  standard deviation of three independent experiments, each performed in duplicate. tk, Thymidine kinase; MMTV, mouse mammary tumour virus.

function by E1A. As Stat2 interacts with p300/CBP, we infer that ISGF3 transactivation function depends, at least in part, upon p300/CBP.

Our results could also be explained by E1A-mediated inhibition of the synthesis and/or nuclear translocation of ISGF3 components. To explore this possibility, we asked whether E1A would repress the transactivation function of GAL4-Stat2 (Fig. 4b). GAL4-Stat2 transactivation was repressed ~eightfold by E1A 12S and by the E1A $\Delta$ 121-150 mutant, but to a lesser extent by the E1A N-terminal and CR1 mutants. This implies that E1A represses GAL4-Stat2 transactivation by binding to and inhibiting p300/CBP. Moreover, the transactivation function of a GAL4-Stat2 $\Delta$ -VP16 fusion protein, in which the p300/CBP binding domain of Stat2 was replaced by the VP16 transactivation domain, (thus allowing it to target the basal transcription machinery directly<sup>17</sup>), was not inhibited by E1A (Fig. 4c). The results of this experiment served to control for: (1) protein synthesis, both GAL4 fusion proteins being constitutively expressed; (2) nuclear translocation, which is dependent on the GAL4 nuclear localization signal; and (3) the integrity of the general transcription machinery in E1A-containing cells. E1A-mediated inhibition of Stat2 and ISGF3 transactivation potential is therefore likely to be mediated primarily by the binding of E1A to p300/CBP.

These results indicate that the Stat2 C-terminal domain, essential for Stat2 and ISGF3 transactivation<sup>6</sup>, functionally interacts with p300/CBP and contributes to IFN- $\alpha$  nuclear signalling. E1A binds to, and inhibits p300/CBP, thereby down-modulating transactivation of Stat2 and ISGF3. This blocks the cellular response to IFN- $\alpha$ . E1A-mediated inhibition of ISGF3 transactivation is also likely to result in reduced synthesis of the components of ISGF3<sup>3,4</sup>, which are transcriptionally regulated by IFN- $\alpha$  (refs 18-21). In summary, our results indicate that there is a novel mechanism for the inhibition of the IFN- $\alpha$ -activated antiviral response by E1A, and strengthen the concept that E1A binding to a p300/CBP family member contributes to adenovirus survival in its natural host. □

## Methods

**Expression screening.** GST-CH1 was labelled with <sup>32</sup>P and used for expression screening of a HeLa  $\lambda$ gt11 library (Clontech) as described<sup>22</sup>.

**In vitro binding reactions.** DNA templates for the synthesis of Stat2 670-851 and Stat2 419-851 were polymerase chain reaction (PCR) products. Stat2 1-851, Stat2 1-712, and Stat2 1-768 were synthesized from intact pBS113, or pBS113 cleaved with *Afl*III or *Nsi*I, respectively. Stat1 was synthesized from pBS91. GST-CH1 or GST-CH1 $\Delta$  immobilized on glutathione-agarose beads were incubated with *in vitro* translated Stat2 products diluted in Hyb75 + 1% milk<sup>24</sup> for 4 h at 4 °C, and washed with EBC (50 mM Tris-HCl, pH 8.0, 240 mM NaCl, 0.5% Nonidet P-40).

**Co-immunoprecipitation.** HeLa cells were exposed to IFN- $\gamma$  (12.5 ng ml<sup>-1</sup>) for 16 h, labelled with <sup>35</sup>S methionine (900  $\mu$ Ci ml<sup>-1</sup>) for 3 h, and stimulated with IFN- $\alpha$  (10,000 units ml<sup>-1</sup>; Schering) for 45 min. Immunoprecipitation and re-immunoprecipitation were performed as described<sup>23,24</sup> (see Supplementary Information). U-2 OS cells were stimulated with IFN- $\alpha$  (5,000 units ml<sup>-1</sup>) for 18 h. Immunoprecipitation reactions (see Supplementary Information) contained 1.27 mg cell lysate and 12  $\mu$ g antibody. Immunoprecipitated proteins were resolved by SDS-PAGE and electroblotted onto Immobilon P (Millipore). A cocktail of mouse monoclonal antibodies to p300/CBP (AC238, AC26, RW128 and RW105), followed by sheep anti-MIG-HRP, was used to detect p300/CBP in the immunoprecipitates by enhanced chemiluminescence (Amersham).

**Plasmids.** The GAL4 DNA-binding domain in pCMXGAL4 was fused to Stat2 residues 670-851 (GAL4-Stat2) and 670-768 (GAL4-Stat2 $\Delta$ ). The VP16 transactivation domain (residues 411-490)<sup>25</sup> was fused to Stat2 residue 768 in GAL4-Stat2 $\Delta$  to create GAL4-Stat2 $\Delta$ -VP16. Plasmid 3 $\Delta$ 51-luc contains an ISRE (residues -206/-87 of the human 2'-5'-oligo(A) synthetase enhancer<sup>26</sup>) upstream of luciferase. For construction details, see Supplementary Information.

**Transfections.** U-2 OS cells were transfected using calcium phosphate<sup>27</sup> in 24-well plates, except where indicated. Luciferase and chloramphenicol acetyltransferase (CAT) activities were normalized for transfection efficiency, as measured by LacZ activity. The total plasmid dose was brought up to 0.75  $\mu$ g with Bluescript DNA in the luciferase assays.

**GAL4-luciferase experiments.** Transfections contained 0.002  $\mu$ g CMV-GAL4-fusion protein-encoding plasmids, 0.35  $\mu$ g CMV-LacZ, and 0.1  $\mu$ g 3  $\times$  GAL4-luc. Experiments using E1A plasmids contained 0.01  $\mu$ g of either CMV vector (backbone), CMV-E1A12S (ref. 16), or CMV-E1A mutant expression plasmids; E1A $\Delta$ 2-36 (ref. 28), E1A $\Delta$ 30-85 (ref. 29) or E1A $\Delta$ 121-150 (ref. 16).

**GAL4-CAT experiments.** Transfections (in 10-cm plates) contained 1  $\mu$ g CMV-LacZ; 5  $\mu$ g pG5CAT; 0.01  $\mu$ g GAL4-Stat2; and 5  $\mu$ g CMVp300-VP16 or CMVp300 $\Delta$ CH1-VP16. The control transfection contained an equimolar amount (2.5  $\mu$ g) of CMV backbone vector.

**ISRE-luciferase experiments.** Transfection mixtures contained 0.1  $\mu$ g 3 $\Delta$ 51-Luc; 0.35  $\mu$ g CMV-LacZ; and 0.01  $\mu$ g of either CMV vector (backbone),

CMV-E1A12S (ref. 16), or CMV-E1A mutant expression plasmids. Cells were stimulated 24 h after transfection with 5,000 Units ml<sup>-1</sup> of IFN- $\alpha$  (Schering) for 6 h. E1A did not significantly affect basal transcription (data not shown).

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1. Darnell, J. E., Kerr, I. M. & Stark, G. R. *Science* **264**, 1415–1421 (1994).
2. Landofo, S., Gribaudo, G., Angeretti, A. & Gariglio, M. *Pharmacol. Ther.* **65**, 415–442 (1995).
3. Kalvakolanu, D. V., Bandyopadhyay, S. K., Harter, M. L. & Sen, G. C. *Proc. Natl Acad. Sci. USA* **88**, 7459–7463 (1991).
4. Gutch, M. J. & Reich, N. C. *Proc. Natl Acad. Sci. USA* **88**, 7913–7917 (1991).
5. Akrill, A. M. et al. *Nucleic Acids Res.* **19**, 4387–4393 (1991).
6. Qureshi, S. A., Leung, S., Kerr, I. M., Stark, G. R. & Darnell, J. E. *Mol. Cell. Biol.* **16**, 288–293 (1996).
7. Kwok, R. P. et al. *Nature* **370**, 223–226 (1994).
8. Bannister, A. J. & Kouzarides, T. *EMBO J.* **14**, 4758–4762 (1995).
9. Kee, B. I., Arias, J. & Montminy, M. R. *J. Biol. Chem.* **271**, 2373–2375 (1996).
10. Kamei, Y. et al. *Cell* **85**, 403–414 (1996).
11. Dai, P. et al. *Genes Dev.* **10**, 528–540 (1996).
12. Arany, Z., Newsome, D., Oldread, E., Livingston, D. M. & Eckner, R. *Nature* **374**, 81–84 (1995).
13. Lundblad, J. R., Kwok, R. P., Laurance, M. E., Harter, M. L. & Goodman, R. H. *Nature* **374**, 81–84 (1995).
14. Leung, S., Qureshi, S. A., Kerr, I. M., Darnell, J. E. & Stark, G. R. *Mol. Cell. Biol.* **15**, 1312–1317 (1995).

15. Bayley, S. T. & Mymrik, J. S. *Int. J. Oncol.* **5**, 425–444 (1994).
16. Stein, R. W., Corrigan, M., Yaciuk, P., Whelan, J. & Moran, E. J. *Virology* **64**, 4421–4427 (1990).
17. Goodrich, J. A., Cutler, G. & Tjian, R. *Cell* **84**, 825–830 (1996).
18. Kumar, R. & Korutla, L. *Exp. Cell Res.* **216**, 143–148 (1995).
19. Yan, R., Qureshi, S., Zhong, Z., Wen, Z. & Darnell, J. E. *Nucleic Acids Res.* **23**, 459–463 (1995).
20. Pine, R., Canova, A. & Schindler, C. *EMBO J.* **13**, 158–167 (1994).
21. Muller, M. et al. *EMBO J.* **12**, 4221–4228 (1993).
22. Kaelin, W. G. J. et al. *Cell* **70**, 351–364 (1992).
23. Schindler, C., Shuai, K., Prezioso, V. R. & Darnell, J. E. *Science* **257**, 809–813 (1992).
24. Krek, W. et al. *Cell* **78**, 161–172 (1994).
25. Triezenberg, S. J., Kingsbury, R. C. & McKnight, S. L. *Genes Dev.* **2**, 718–729 (1988).
26. Cohen, B., Peretz, D., Vaiman, D., Benech, P. & Chebath, J. *EMBO J.* **7**, 1411–1419 (1988).
27. Chen, C. & Okayama, H. *Mol. Cell. Biol.* **7**, 2745–2752 (1987).
28. Kraus, V. B., Moran, E. & Nevins, J. R. *Mol. Cell. Biol.* **12**, 4391–4399 (1992).
29. Whyte, P., Williamson, N. M. & Harlow, E. *Cell* **56**, 67–75 (1989).

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## Rhodopsin activation blocked by metal-ion-binding sites linking transmembrane helices C and F

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**A LARGE superfamily of receptors containing seven transmembrane (TM) helices transmits hormonal and sensory signals across the plasma membrane to heterotrimeric G proteins at the cytoplasmic face of the membrane. To investigate how G-protein-coupled receptors work at the molecular level, we have engineered metal-ion-binding sites between TM helices to restrain activation-induced conformational change in specific locations. In rhodopsin, the photoreceptor of retinal rod cells, we substituted histidine residues for natural amino acids at the cytoplasmic ends of the TM helices C and F. The resulting mutant proteins were able to activate the visual G protein transducin in the absence but not in the presence of metal ions. These results indicate that the TM helices C and F are in close proximity and suggest that movements of these helices relative to one another are required for transducin activation. Thus a change in the orientations of TM helices C and F is likely to be a key element in the mechanism for coupling binding of ligands (or isomerization of retinal) to the activation of G-protein-coupled receptors.**

G-protein-coupled receptors (GPCRs) activate G proteins by catalysing exchange of GTP for GDP bound to the G-protein  $\alpha$ -subunit, triggering regulation (by the GTP-bound  $\alpha$ -subunit,  $\alpha$ -GTP, and the free complex of  $\beta\gamma$  subunits) of effector enzymes or ion channels that initiate neural, cardiovascular, endocrine or sensory responses<sup>1,2</sup>. To understand in molecular detail how GPCRs communicate with G proteins will require three-dimensional structures of all the protein components and

identification of the key changes in their conformations. The heterotrimeric complex of the visual G protein transducin has been analysed to a resolution of 2.0 Å (refs 3, 4), and the structure of a second heterotrimer,  $G_{\alpha\beta\gamma}$ , was reported at lower resolution<sup>5</sup>. For GPCRs, however, only projection density maps at ~6.0 Å resolution are available for rhodopsin<sup>6,7</sup>. Until well-defined GPCR structures become available, valuable information can come from experiments that define distances between the TM helices, modulate their movements, and specifically alter G-protein activation. Engineering of metal-ion-binding sites represents one such strategy, previously used with success to modulate specific properties and functions of proteins in a metal-dependent manner<sup>8,9</sup>. The small size and geometrically precise interactions of metal ions provide important information regarding the spatial orientation and proximity of the amino acids involved<sup>10</sup>.

We applied this approach to rhodopsin, an especially well-characterized GPCR. In rhodopsin, photoconversion of the 11-*cis* chromophore to the all-*trans* configuration activates the receptor<sup>11</sup>, enabling it to catalyse guanine-nucleotide exchange by transducin<sup>12</sup>. A model<sup>13</sup> for the packing of the seven transmembrane helices, derived from sequence analysis and interpretation of two-dimensional rhodopsin arrays<sup>6,7</sup>, locates TM helix C in the middle of the molecule. Mutagenesis, spectroscopy and site-directed spin labelling point to TM helices C, F and G in rhodopsin as key elements in transmitting the signal of retinal isomerization to the cytoplasmic domain of the receptor<sup>14–17</sup>. To restrain potential conformational changes, we sought to link these helices with engineered metal-ion-binding sites, created by substituting histidines (which coordinate transition metals<sup>18</sup>) at specific sites.

Figure 1a shows the positions of the histidine residues we substituted at the cytoplasmic ends of TM helices C, F and G. We chose individual residues for such substitution by reference to a schematic model (O.L., manuscript in preparation), similar to a previous model<sup>13</sup>, in which these residues point towards the inside of the rhodopsin molecule (Fig. 1b). Avoiding mutation of highly conserved residues, except for Glu 134, we constructed double and triple histidine mutants in TM helices C and F (E134H–K248H/R252H, V138H–T251H, V138–K248/R252H and V138H/K141H–T251H) or C and G (V138H–N310–Q112H). In the presence or absence of Zn(II), we tested the ability of each of these five mutant rhodopsins, plus two controls (V138H and T251H), to activate the  $\alpha$ -subunit of transducin ( $\alpha_t$ ). Two rather different activation assays, performed using cell membranes (Fig. 2) or in detergent solution (Fig. 3), produced the same result: one set of histidine substitutions linking TM helices C and F (via

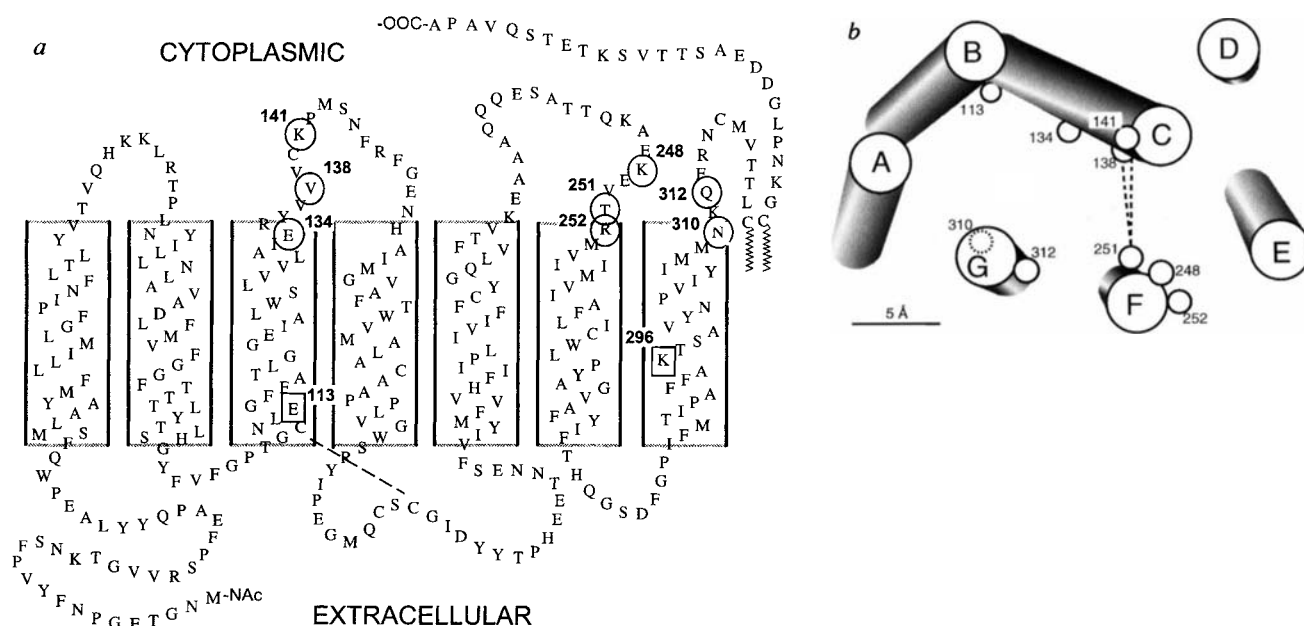


FIG. 1 *a*, Secondary structural model of bovine rhodopsin. The protein's cytoplasmic and extracellular loops are depicted above and below the transmembrane helices, respectively. Boxes indicate sites of the retinal-chromophore Schiff-base linkage (Lys 296) and the Schiff-base counterion (Glu 113). Circles indicate residues replaced by histidines using site-directed mutagenesis. The seven mutant opsins included in this study are: (1) V138H-T251H; (2) V138H/K141H-T251H; (3) V138H-K248H/

R252H; (4) V138H-N310H/Q312H; (5) E134H-K248H/R252H; (6) V138H, and (7) T251H. *b*, Model of the seven-TM helix arrangement in rhodopsin<sup>6,7,13</sup> showing probable locations of the mutated residues. The residues are indicated by circles and numbers. The Schiff-base counterion at position 113 is also indicated. Helices A-G are depicted in the plane of the membrane, as viewed from the cytoplasm.

residues 138 and 251), but not other potential links, prevents  $\alpha_t$  activation in the presence of Zn(II).

The first assay used microsomes of COS-7 cells transiently expressing the mutant rhodopsin and wild-type  $\alpha_t$ ,  $\beta_1$  and  $\gamma_1$ , to test rhodopsin's ability to promote binding of GTP- $\gamma$ S to  $\alpha_t$ , assessed by measuring GTP- $\gamma$ S-dependent protection of a specific proteolytic band, as described<sup>19</sup>. In the presence of all-*trans*-retinal and GTP- $\gamma$ S under ambient light, addition of 10  $\mu$ M Zn(II) nearly abolished  $\alpha_t$  activation by two mutant opsins, V138H-T251H and

V138H/K141H-T251H, as indicated by trypsin digestion of  $\alpha_t$  fragments (Fig. 2*a*). The protected  $\alpha_t$  band could be rescued by chelating the zinc ions with EDTA (not shown). In contrast, Zn(II) had no effect on the ability of the other mutants to protect  $\alpha_t$  from degradation; all protected  $\alpha_t$  as well as wild-type rhodopsin did (Fig. 2*b*). These results indicate that the  $\alpha$ -carbons of residues 138 and 251 are separated by no more than 13 Å, the maximal distance at which histidine substitutions at these positions could coordinate a metal ion<sup>20</sup>.

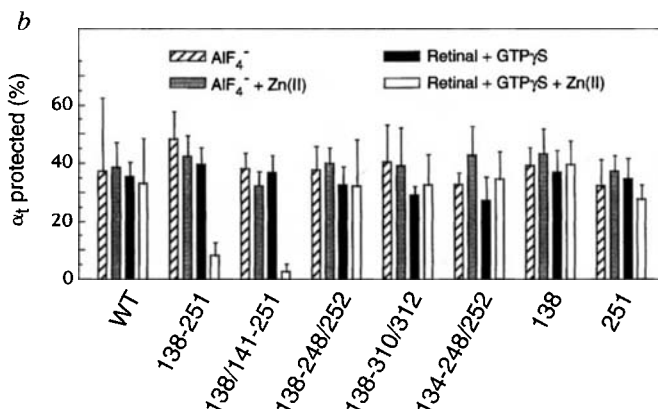
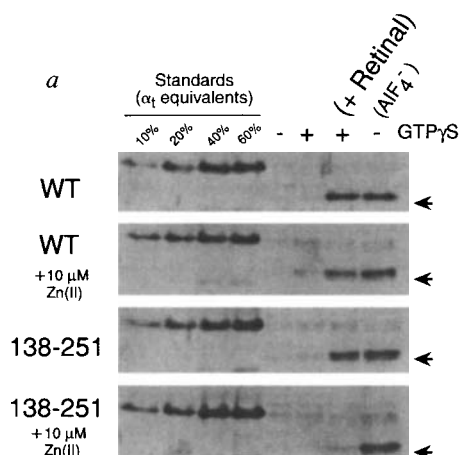
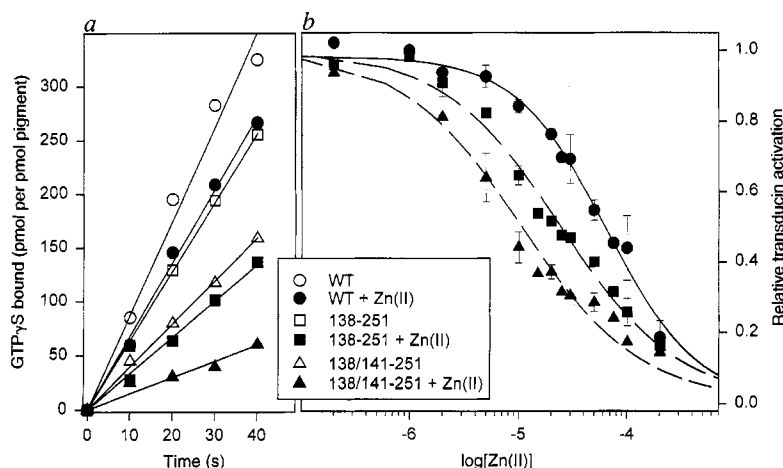


FIG. 2 Effects of Zn(II) on activation of transducin by wild-type and mutant rhodopsins. *a*, Activation was assessed as described<sup>19</sup>, by measuring the appearance of trypsin-resistant  $\alpha_t$  fragments (arrows), which reflect activation-induced conformational change. In each immunoblot, the four lanes on the left represent percentage equivalents (10, 20, 40 and 60%) of no-trypsin control samples; the four lanes on the right represent (from left to right) samples incubated with no addition, GTP- $\gamma$ S alone, GTP- $\gamma$ S plus

all-*trans*-retinal, or AIF<sub>4</sub><sup>-</sup>, all at 20 °C for 4 min; these four samples were then treated with TPCK trypsin for 1 h at 4 °C before reactions were terminated by addition of aprotinin and PMSF<sup>19</sup>. *b*, Quantification of replicate experiments testing effects of Zn(II) on  $\alpha_t$  activation by wild-type and the indicated mutant rhodopsins in the presence of AIF<sub>4</sub><sup>-</sup> or retinal plus GTP- $\gamma$ S. Each bar indicates the mean  $\pm$  2 s.e.m. of at least 4 determinations in 2–5 separate experiments.

FIG. 3 Transducin activation by purified mutant pigments was assayed using a GTP- $\gamma$ S filter-binding assay. Wild-type (WT) rhodopsin and mutant pigments V138H-T251H and V138H/K141H-T251H are shown. *a*, Bound GTP- $\gamma$ S plotted as a function of time in the absence (open symbols) or presence of 30  $\mu$ M Zn(II) (filled symbols). *b*, Relative transducin activation plotted as a function of the log of the Zn(II) ion concentration. Half-maximal inhibitory concentration ( $IC_{50}$ ) values for inhibition by Zn(II) were determined from the fits of these curves to be 25  $\mu$ M for V138H-T251H, 10.5  $\mu$ M for V138H/K141H-T251H, and 68  $\mu$ M for rhodopsin.



A second set of experiments (Fig. 3) used transducin binding of radiolabelled GTP- $\gamma$ S in a filter-binding assay to test the ability of solubilized mutant pigments to catalyse light-dependent guanine-nucleotide exchange<sup>21</sup>. Each mutant was reconstituted with 11-*cis*-retinal and purified in dodecyl maltoside detergent before measuring light-dependent transducin activity in solution. At 30  $\mu$ M, Zn(II) caused a 50% decrease in the activation of transducin by the same two mutant pigments (V138H-T251H and V138H/K141H-T251H) whose activities were blocked by Zn(II) in the microsome assay (Fig. 3a). Figure 3b shows that inhibition of transducin activation by the mutant opsins depended on the concentration of added zinc, with apparent  $IC_{50}$  values of 10.5  $\mu$ M for V138H/K141H-T251H and 25.0  $\mu$ M for V138H/T251H. These apparent inhibition constants are comparable to those measured in other designed metal sites<sup>8,9</sup>. Addition of the extra histidine at position 141 increased the apparent binding affinity for Zn(II), presumably because a tighter metal-binding site was created (by the three histidines acting together, or because the added histidine created a second metal-binding site with higher affinity).

A third mutant opsin (V138H-K248H/R252H), was not affected by Zn(II) in the microsomal assay (Fig. 2b) but was significantly inhibited by Zn(II) in detergent micelles, with a half-maximal inhibitory concentration ( $IC_{50}$ ) value of 28  $\mu$ M (not shown); another mutant opsin (E134H-K248H/R252H) was significantly defective in its ability to activate transducin in the absence of Zn(II) in the detergent-soluble assay (13% of wild-type activity; not shown), but not in the microsomal assay (Fig. 2b). Although the reason for the discrepancy between the two assays is unknown, they could result from increased flexibility of the rhodopsin conformation in detergent micells compared with the lipid environment of COS cell microsomes<sup>17</sup>. Finally, wild-type rhodopsin showed some sensitivity ( $IC_{50}$  = 68  $\mu$ M) to Zn(II) in the detergent-solubilized GTP- $\gamma$ S filter-binding assay (Fig. 3), an effect that may in part be due to the ability of Zn(II) to inhibit receptor-independent  $AlF_4^-$ -induced activation of transducin ( $IC_{50}$ , 225  $\mu$ M; results not shown).

As depicted for wild-type and one mutant rhodopsin (Fig. 4), addition of Zn(II) did not affect the spectral properties of any of the pigments tested. Like wild-type, each mutant opsin bound 11-*cis*-retinal to form a stable pigment with an absorption peak at 500 nm. As shown for V138H/K141H-T251H and wild-type rhodopsin (Fig. 4), the presence of Zn(II) did not affect these ground-state spectral properties. Even in mutants (like V138H/K141H-T251H;

Fig. 4) whose ability to activate transducin was strongly inhibited by Zn(II), the metal did not interfere with the ability of the opsins to form the metarhodopsin II (MII) spectral form upon illumination. Thus Schiff base deprotonation was not affected by Zn(II). Spectral properties of the mutant pigments were indistinguishable from those of wild-type rhodopsin, even in the presence of Zn(II) (up to 5 mM).

The results shown in Figs 2 and 3 compared with Fig. 4 show that the metal-binding sites blocked transducin activation without directly affecting the retinal-binding pocket or light-induced Schiff-base deprotonation. This difference is especially important as these two helices are involved in formation of the retinal-binding pocket<sup>22</sup>; a rhodopsin mutation that uncouples Schiff-base deprotonation from the pigment's ability to activate transducin has been reported<sup>23</sup>.

Thus our results indicate that the cytoplasmic ends of TM helices C and F are in proximity in rhodopsin and suggest that their relative positions must change in order to form the conformation of rhodopsin required to activate transducin. An alternative possibility, that Zn(II) imposes a steric block which prevents transducin binding, is less likely (see below). These conclusions advance our understanding, based on mutagenesis and biophysical studies<sup>14,15,17,24,25</sup>, that cytoplasmic ends of these two helices and

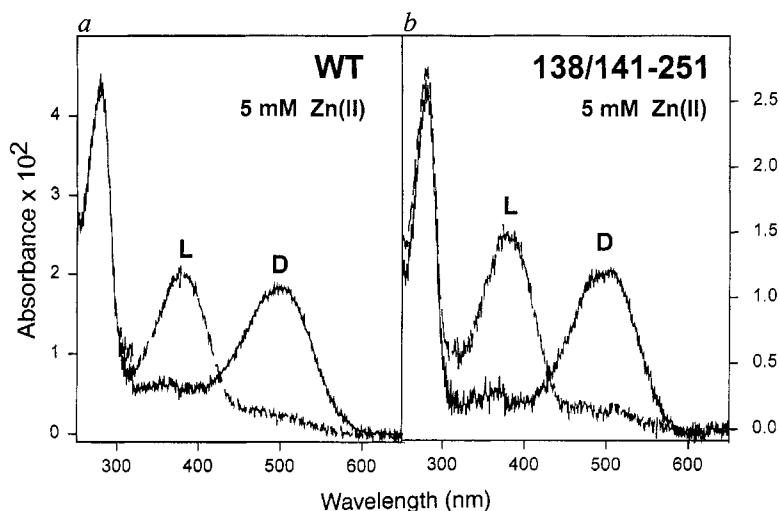


FIG. 4 Ultraviolet-visible absorption spectra of recombinant pigments. The spectra are shown of wild-type (WT) bovine rhodopsin (*a*) and mutant pigment V138H/K141H-T251H (*b*) purified in dodecyl maltoside detergent. Dark spectra (D) and spectra after illumination (L) were recorded in the presence of 5 mM Zn(II). Each of the seven mutant pigments displayed a  $\lambda_{max}$  value of 500 nm, which shifted to 380 nm upon illumination.



parts of adjacent cytoplasmic loops play key roles in G-protein activation by GPCRs. Glu 113 and Glu 134, both located in TM helix C, are important in forming the active state of rhodopsin<sup>26–28</sup>. Electron paramagnetic resonance (EPR) spin-labelled residues on the cytoplasmic membrane surface of TM helix C experience perturbation of their steric environments, consistent with movement of this region upon formation of the active rhodopsin photoproduct, MII (ref. 15). The hydrogen-bond strength of Glu 122 (TM helix C) changes upon MII formation<sup>29</sup>. Tryptophan residues at positions 126 (TM helix C) and 265 (TM helix F) experience changes in their environments upon MII formation<sup>22</sup>. In addition, Gly 121 and Phe 261 form a complementary contact site in rhodopsin that is involved in the functional interaction between TM helices C and F (M. Han, S. W. Lin, S. O. Smith and T.P.S., manuscript submitted; M. Han, S.W. Lin, M. Minkova, S. O. Smith and T. P. Sakmar, manuscript submitted).

Taken together with previous findings, our results indicate direct coupling of receptor activation to a change in the spatial disposition of TM helices C and F. This could occur if movement of TM helices C and F in turn changes the conformation of the connected intracellular loops (especially loop EF) that contribute to binding surfaces and tertiary contacts of rhodopsin with transducin<sup>17,24</sup>. Finally, because the overall arrangement of TM helices is evolutionarily conserved and shared among GPCRs, motion of helices C and F relative to one another may be a part of the activation mechanism of all G-protein-coupled receptors. Given the structural and functional similarities among rhodopsin and other GPCRs, it is likely that these helix-helix interactions are modulated by ligand binding, and in turn transmit an intramolecular signal from the ligand-binding pocket, embedded in the PM, to the cytoplasmic surface of the receptor.

During the preparation of this manuscript we learned that Farrens *et al.* (ref. 30) have used a different experimental approach to reach a similar conclusion. They used cysteines substituted in TMs C and F (at sites close to those we tested) in spin-label and disulphide crosslinking experiments. These results indicate that movement of these two TMs is involved in transducin activation. □

## Methods

Rhodopsin mutations, expression in COS cells, and quantification of trypsin protection were performed as described<sup>19</sup>, except that microsomal fractions were reconstituted in 250 mM sucrose, 5 mM Tris-HCl (pH 7.4) without reducing agent, and diluted into reaction mixtures in a buffer containing 20 mM HEPES (pH 7.4), 145 mM NaCl, 2 mM MgCl<sub>2</sub> and 0.1 mM CaCl<sub>2</sub>.

The ability of each purified mutant pigment to activate transducin in the absence or presence of Zn(II) was evaluated by a GTP-γS filter-binding assay in dodecyl maltoside detergent micelles<sup>21</sup>. The amount of GTP-γS bound to a filter was plotted as a function of time after illumination, and a linear regression was used to determine the rate of transducin activation. The absorption at 500 nm was used to determine the amount of pigment assayed.

For analysis of UV-visible absorption spectra, membranes from COS cells expressing recombinant pigments were treated with 11-*cis*-retinal and the pigments were purified in dodecyl maltoside detergent buffer in darkness, as described<sup>21</sup>. Illumination was with a 150-watt light source through a 495 nm long-pass filter for 10 s (ref. 21).

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- Bourne, H. R., Sanders, D. A. & McCormick, F. *Nature* **363**, 274–276 (1990).
- Freissmuth, M., Casey, P. J. & Gilman, A. G. *FASEB J.* **3**, 2125–2131 (1989).
- Noel, J. P., Hamm, H. E. & Sigler, P. B. *Nature* **366**, 654–663 (1993).
- Lambright, D. G. *et al.* *Nature* **379**, 311–319 (1996).
- Wall, M. A. *et al.* *Cell* **83**, 1047–1058 (1995).
- Schertler, C. F. X. & Hargrave, P. A. *Proc. Natl Acad. Sci. USA* **92**, 11578–11582 (1995).
- Schertler, C. F. X., Villa, C. & Henderson, R. *Nature* **362**, 770–772 (1993).
- Elling, C. E., Nielsen, S. M. & Schwartz, T. W. *Nature* **374**, 74–77 (1995).
- Willet, W. S., Gilmore, S. A., Perona, J. J., Fletterick, R. J. & Craik, C. S. *Biochemistry* **34**, 2172–2180 (1995).
- Regan, L. *Trends Biochem. Sci.* **20**, 280–285 (1995).
- Yoshizawa, T. & Wald, G. *Nature* **197**, 1279–1286 (1963).
- Kibelbek, J., Mitchell, D. C., Beach, J. M. & Litman, B. L. *Biochemistry* **30**, 6761–6768 (1991).
- Baldwin, J. M. *EMBO J.* **12**, 1693–1703 (1993).
- Oprian, D. D., Molday, R. S., Kaufman, R. J. & Khorana, H. G. *Proc. Natl Acad. Sci. USA* **84**, 8874–8878 (1987).
- Farahbakhsh, Z. T., Ridge, K. D., Khorana, H. G. & Hubbell, W. L. *Biochemistry* **34**, 8812–8819 (1995).

- Zyaga, T. A., Min, K. C., Beck, M. & Sakmar, T. P. *J. Biol. Chem.* **268**, 4661–4667 (1993).
- Frank, R. R., Sakmar, T. P., Graham, R. M. & Khorana, H. G. *J. Biol. Chem.* **267**, 9478–9480 (1992).
- Chakrabarti, P. *Protein Eng.* **4**, 57–63 (1991).
- Garcia, P. D., Onrust, R., Bell, S. M., Sakmar, T. P. & Bourne, H. R. *EMBO J.* **14**, 4460–4469 (1995).
- Higaki, J. N., Fletterick, R. J. & Craik, C. S. *Trends Biochem. Sci.* **17**, 100–104 (1992).
- Min, K. C., Zyaga, T. A., Cypess, A. M. & Sakmar, T. P. *J. Biol. Chem.* **268**, 9400–9404 (1993).
- Lin, S. W. & Sakmar, T. P. *Biochemistry* **35**, 11149–11159 (1996).
- Zyaga, T. A., Fahmy, K. & Sakmar, T. P. *Biochemistry* **33**, 9753–9761 (1994).
- Frank, R. R., König, B., Sakmar, T. P., Khorana, H. G. & Hofmann, K. P. *Science* **250**, 123–125 (1990).
- Liu, J., Conklin, B. R., Blin, N., Yun, J. & Wess, J. *Proc. Natl Acad. Sci. USA* **92**, 11642–11646 (1995).
- Fahmy, K., Siebert, F. & Sakmar, T. P. *Biophys. Chem.* **56**, 171–181 (1995).
- Fahmy, K. & Sakmar, T. P. *Biochemistry* **32**, 7229–7236 (1993).
- Cohen, G. B., Oprian, D. D. & Robinson, P. R. *Biochemistry* **31**, 12592–12601 (1992).
- Fahmy, K. *et al.* *Proc. Natl Acad. Sci. USA* **92**, 7229–7236 (1993).
- Farrens, D. L., Altenback, C., Yang, K., Hubbell, W. L. & Khorana, H. G. *Science* (in the press).

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## Neutralizing antibody to human rhinovirus 14 penetrates the receptor-binding canyon

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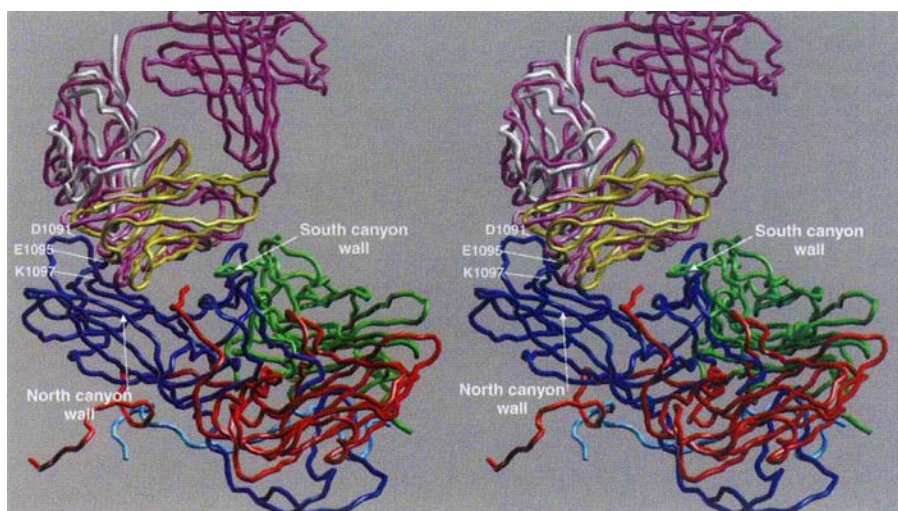
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**THE three-dimensional structure of intact human rhinovirus 14 (HRV-14) complexed with Fab fragments (Fab17-IA) from a strongly neutralizing antibody that binds bivalently to the virion<sup>1,2</sup> has been determined to 4.0 Å resolution by a combination of X-ray crystallography and cryo-electron microscopy. In contradiction to the most commonly held model of antibody-mediated neutralization, Fab17-IA does not induce a conformational change in the HRV-14 capsid. Instead, the paratope of the antibody undergoes a large conformational change to accommodate the epitope. Unlike any previously described antibody-antigen structure, the conserved framework region of the antibody makes extensive contact with the viral surface. Fab17-IA penetrates deep within the canyon in which the cellular receptor for HRV-14 binds<sup>3,4</sup>. Hence, it is unlikely that viral quaternary structure evolves merely to evade immune recognition. Instead, the shape and position of the receptor-binding region on a virus probably dictates receptor binding and subsequent uncoating events and has little or no influence on concealing the virus from the immune system.**

The capsid of HRV-14 is composed of 60 copies of four viral proteins, VP1–VP4. Each of the first three proteins has a relative molecular mass of ~30,000 ( $M_r$  ~30K) and a similar β-barrel structure. The smaller VP4 ( $M_r$  ~7K) lies at the capsid–RNA interface with an extended structure<sup>3</sup>. The monoclonal antibody 17-IA binds to the N1m-IA site which was defined by natural escape mutations at residues D1091 and E1095 on the B–C loop of VP1 (the first digit designates the viral protein and the remaining three designate the residue number). The N1m-IA epitope lies at the ‘north rim’ of a depression (canyon) on the viral surface that encircles each of the five-fold icosahedral axes of the virus<sup>3</sup> and is where the receptor, ICAM-1, binds<sup>4</sup>.

The variable domains (VH–VL) of Fab17-IA make extensive contact with both the north and south walls of the canyon region. At the north wall, the antigen-binding region (paratope) contacts ~550 Å<sup>2</sup> of the viral surface around N1m-IA site (Fig. 1). The

FIG. 1 C $\alpha$  backbone of the initial and final models. VP1 is blue, VP2 green, VP3 red, initial Fab purple, final VL domain white, and final V<sub>H</sub> domain yellow. The RNA interior of the virus is towards the bottom of the figure, and some key contact residues around the HRV14 NIm-1A site are labelled.



heavy-chain hypervariable region dominates the contact surface: 23 residues from the heavy chain but only 9 residues from the light chain. As was proposed on the basis of cryo-electron microscopy on this HRV/Fab complex<sup>5</sup>, coulombic interactions dominate the paratope–epitope interface with roughly two-thirds of the total buried viral surface being contributed by charged side chains. Fab17-1A contacts  $\sim 49$ , 12 and  $0.3 \text{ \AA}^2$  of the K1097, K1085 and K1236 side chains, respectively. These contact areas correlate with a decrease in Fab affinity by 44-, 9.3- and 0.8-fold, respectively, when residues K1097, K1085 and K1236 are separately mutated to glutamate<sup>5</sup>. Additional charge interactions include Arg 91<sup>L</sup> (Fab17 numbering corresponds to Kabat<sup>6</sup>: the last letter designates the chain type) which forms salt bridges with the two natural escape mutation sites (Glu 1095, Asp 1091), and Asp 54<sup>H</sup> and Asp 56<sup>H</sup> interdigitate between Arg 1094 and Lys 1097. Four residues, His 1078, Val 1079, Thr 1080 and Asp 1101, which are all considered to lie at the bottom of the north side of the canyon, contact the bound Fab17. Thus, these interactions provide strong evidence that antibodies are capable of binding deep into viral crevices.

The south wall of the canyon ( $\sim 300 \text{ \AA}^2$ ) is also contacted by Fab17-1A. This region is nearly identical to that recognized by the HRV-14 receptor ICAM-1 (ref. 4) and includes a fairly even distribution of charged, polar and hydrophobic residues. Approximately one-third of this area involves contacts with heavy-chain CDR3 residues (where CDR is complement-determining region) that mostly interact with the carboxy terminus of VP3 near the base of the canyon. The remaining  $\sim 200 \text{ \AA}^2$  of the surface contacted by the V<sub>H</sub> domain comprises residues from the conserved framework regions FR1 and FR3. The importance of these framework contacts is suggested by results on another Fab (Fab12-1A) that binds to HRV-14 in the same orientation as Fab17-1A and only differs from Fab17-1A by 5 amino acids (Z. C. Che, T. J. S., N.H.O. and T.S.B., manuscript in preparation). Both monoclonal antibodies probably came from the same mother cell but differ as a result of somatic mutations. Two of the five amino-acid differences between antibodies 12-1A and 17-1A lie within the CDR1 loop (32<sup>H</sup> and 34<sup>H</sup>); the remaining three are in the FR3 region (68<sup>H</sup>, 82a<sup>H</sup> and 76<sup>H</sup>). The first two of these FR3 residues (68<sup>H</sup> and 82a<sup>H</sup>) contact the south wall of the canyon. Although somatic mutations occur throughout the variable domain<sup>7</sup>, it is unlikely that mutations in these framework sites that contact the viral surface are merely coincidental.

Several large changes in the Fab17-1A structure accompany binding to HRV-14, although the virus capsid remains essentially rigid (Fig. 2a). A large conformational change occurs in the heavy-chain CDR3 loop (Fig. 2b, c, d) where the C $\alpha$  atom of Tyr102<sup>H</sup> moves up to  $5.6 \text{ \AA}$  from its position in the unliganded Fab

structure (Fig. 2b). This movement opens the cleft between the heavy- and light-chain hypervariable regions (Fig. 2c, d) in a manner quite similar to that in the heavy-chain CDR3 loop of other Fab/antigen complexes<sup>8</sup> and generates additional space for Arg 91<sup>L</sup> to move further into the cleft, where it forms salt bridges with Asp 1091 and Glu 1095. The only changes in the HRV-14 capsid around the epitope are confined to the side chains on the NIm-1A loop that contact the Fab.

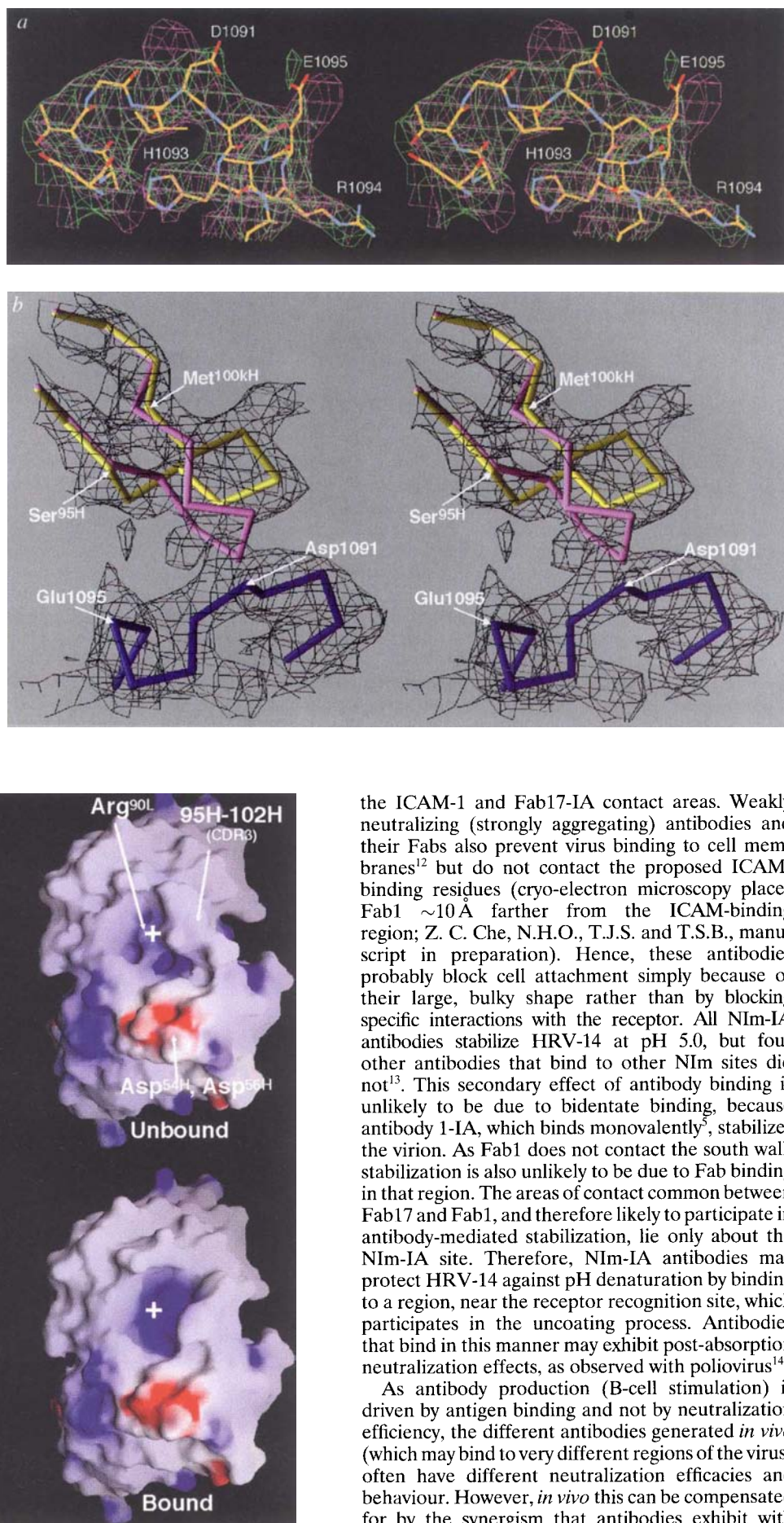
Removed from the Fab-virus contacts ( $>12 \text{ \AA}$  away), unassigned electron density was observed inside the HRV-14 drug-binding cavity and this distorts the canyon floor just as WIN compounds do<sup>9</sup>. We attribute this density to the cryoprotectant PEG400 which was present in high concentration (40%) for these crystal diffraction experiments; this density was not observed in the native HRV-14 electron density maps. Alternatively, Fab–virus interactions might facilitate the binding of this small compound but, because conformational changes do not occur at the contact interfaces upon Fab binding, it is unlikely that the canyon floor would be affected by Fab binding. Nevertheless, the appearance of this density raises the possibility that previously observed ‘pocket factors’<sup>10</sup>, which bind to the same site on other serotypes of HRV and give a nearly identical appearance, may have come from the crystallization mother liquor rather than from the host cell.

The canyon hypothesis<sup>3,11</sup> proposes that conserved residues in the floor of the HRV-14 canyon bind to the cell receptor (ICAM-1), but that the small dimensions of the canyon should limit accessibility to antibodies (Fig. 3). Also, HRV-14 morphology should allow residues at the top of the canyon to mutate freely without affecting virus–receptor interactions. Although a number of natural escape mutation sites lie at the upper north rim of the canyon, our crystal structure analysis shows that Fab17-1A contacts extensive regions inside the canyon, including most of those residues involved in receptor recognition. Thus, the lack of mutations elsewhere in the Fab contact region is more likely to be due to the deleterious effects of such mutations, rather than to antibody accessibility. Indeed, mutation of Lys 1097, which lies halfway down the north canyon wall and interacts with Asp 54<sup>H</sup> and Asp 56<sup>H</sup>, to Gln or Glu reduces virus viability in addition to blocking antibody binding.

The structure determination of the HRV–Fab complex clearly shows that strongly neutralizing antibodies do not have to induce large conformational changes in the virus capsid to cause neutralization. If the viral epitope maintains a fairly rigid structure about which the Fab conforms, then what is the mechanism of neutralization? Abrogation of HRV-14 cell attachment by antibody 17-1A is directly correlated with *in vitro* neutralization and is rationalized as a consequence of the extensive overlap between



FIG. 2 Conformational changes at the paratope/epitope interface. *a*, Comparison between NIm-1A loop region in the unliganded HRV-14 (mauve) and the Fab/virus complex (green) electron density maps. The atomic coordinates of the native HRV-14 structure were used to calculate the unliganded HRV-14 electron density map, whereas the electron density map of the virus–Fab complex was calculated using phases from 20-fold real-space averaging. The structure of the liganded structure is coloured according to atom type (carbon, yellow; oxygen, red; nitrogen, blue). *b*, Initial (unliganded Fab structure) and final structures of the heavy-chain CDR3 are purple and yellow, respectively. The NIm-1A site is shown in dark blue. The black cage is the 4 Å electron density map calculated using the experimentally determined phases from 20-fold real-space averaging and phase extension. For this diagram, the  $V_H$  domains of the initial and final models were aligned to each other. *c*, *d*, Stereo diagram of electrostatic potential and structural changes in the hypervariable region before (*c*) and after (*d*) binding to the NIm-1A site. The view is towards the hypervariable region, with the  $V_L$  domain towards the top of the diagram and the  $V_H$  domain towards the bottom. Surfaces of positive and negative potential are depicted blue and red, respectively. The largest conformational changes occur in the heavy-chain CDR3 loop and in the side chain of Arg 91<sup>L</sup>. White crosses provide points of reference in the stereo views.

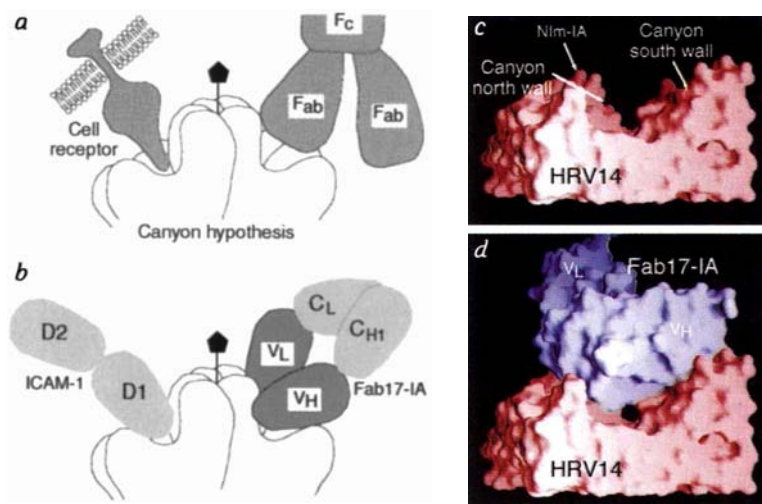


the ICAM-1 and Fab17-1A contact areas. Weakly neutralizing (strongly aggregating) antibodies and their Fabs also prevent virus binding to cell membranes<sup>12</sup> but do not contact the proposed ICAM-binding residues (cryo-electron microscopy places Fab1 ~10 Å farther from the ICAM-binding region; Z. C. Che, N.H.O., T.J.S. and T.S.B., manuscript in preparation). Hence, these antibodies probably block cell attachment simply because of their large, bulky shape rather than by blocking specific interactions with the receptor. All NIm-1A antibodies stabilize HRV-14 at pH 5.0, but four other antibodies that bind to other NIm sites did not<sup>13</sup>. This secondary effect of antibody binding is unlikely to be due to bidentate binding, because antibody 1-1A, which binds monovalently<sup>5</sup>, stabilizes the virion. As Fab1 does not contact the south wall, stabilization is also unlikely to be due to Fab binding in that region. The areas of contact common between Fab17 and Fab1, and therefore likely to participate in antibody-mediated stabilization, lie only about the NIm-1A site. Therefore, NIm-1A antibodies may protect HRV-14 against pH denaturation by binding to a region, near the receptor recognition site, which participates in the uncoating process. Antibodies that bind in this manner may exhibit post-absorption neutralization effects, as observed with poliovirus<sup>14</sup>.

As antibody production (B-cell stimulation) is driven by antigen binding and not by neutralization efficiency, the different antibodies generated *in vivo* (which may bind to very different regions of the virus) often have different neutralization efficacies and behaviour. However, *in vivo* this can be compensated for by the synergism that antibodies exhibit with



FIG. 3 Fab/virus canyon interactions. *a*, The HRV/ICAM-1 and HRV/Fab17-IA complexes, showing the HRV-14 canyon that is accessible to cellular receptor but inaccessible to antibody (adapted from ref. 30). *b*, Current view of virus/receptor and virus/antibody interactions showing that the Fab contacts a large portion of the canyon but not residues at the very bottom (the 'floor'). Molecular surfaces of HRV-14 (*c*) and the Fab17-IA/HRV-14 complex (*d*). The view is parallel to the canyon floor, with an icosahedral 5-fold axis towards the left, the nearest 2-fold axis towards the right, and the RNA interior towards the bottom of the diagram. HRV-14, the variable domains of the bound Fab, and the molecular interfaces are shown in red, blue and grey, respectively. Only a small portion of the canyon floor is not contacted by the bound Fab.



other immune system components. Many different viruses are known in which antibodies neutralize weakly or not at all *in vitro*, yet still yield *in vivo* protection. For example, with foot-and-mouth disease virus (FMDV), antibody-mediated *in vivo* processes such as opsonization (antibody-mediated phagocytosis) and the reticuloendothelial system can play a dominant role in protection<sup>15</sup>.

Our structure determination of the HRV-Fab complex has helped to define the relationship between virus architecture and receptor-binding sites. Despite its recessed receptor-binding region, HRV-14 exhibits similarities to viruses with exposed cell recognition sites: these include FMDV<sup>16,17</sup>, Sindbis virus<sup>18,19</sup>, poliovirus<sup>20</sup>, and the haemagglutinin spike of influenza<sup>21</sup>. It appears that the location and shape of the cell-receptor-binding site on a virus is dictated by the nature of the specific receptor being recognized, as well as what processes occur subsequent to receptor binding. For HRV-14, the canyon does not protect the ICAM-1 binding site from antibody recognition, but does allow ICAM-1 to cause virus uncoating<sup>22</sup>. Finally, as was observed in the case of influenza virus<sup>21</sup>, the surface of the virus covered by the antibody is much larger than that covered by ICAM-1 (ref. 4). Therefore, many residues on the virus offer potential sites for mutation that can thwart antibody binding without affecting receptor binding, thus permitting conserved residues to be exposed to the immune system. □

## Methods

**Crystallization.** HRV-14 was purified as described<sup>23</sup> and Fab fragments were generated from the monoclonal antibody, mAb17-IA, as described<sup>2</sup>. Virus and Fab sample, dialysed against 10 mM Tris, pH 7.5, were combined at a ratio of ~240 Fab molecules per virion and stored at 4 °C for between 12 h and 3 days. The complex was concentrated at 5–10 °C using centrifuge concentrators with a 10K molecular-weight cutoff. The low temperature, low-ionic-strength buffer, and high concentration (10–20 mg ml<sup>-1</sup>) facilitated the precipitation of the Fab/

virus complex and yielded larger crystals than when the complex was concentrated in the presence of high salt. The precipitate was resuspended in and dialysed against 10 mM Tris buffer, pH 7.5, 100 mM NaCl. The solution, at room temperature, was then passed through a 0.2-μ syringe filter and concentrated to ~0.9–1.0 mg ml<sup>-1</sup> extinction coefficient (7.7 ml mg<sup>-1</sup> cm<sup>-1</sup>) using a Centricon 10 filter and centrifuging at 4,000–5,000g and 17–20 °C. The complex was crystallized using the vapour diffusion method, with a reservoir containing 1 ml of 10 mM Tris buffer, pH 7.5, 250–300 mM NaCl, 10 mM CaCl<sub>2</sub>, and 0.75% PEG 8000. Each sitting drop contained 20 μl of the complex to which 20 μl of precipitating buffer (10 mM Tris buffer, pH 7.5, 10 mM CaCl<sub>2</sub> and 0.75% PEG 8000) was added. Crystals with a cubic habit usually appeared in 10–14 days and grew up to 0.8 mm in width within one month.

**Data collection.** Crystals were extremely sensitive to X-ray radiation at room temperature. Consequently, data collection was done with crystals flash-frozen at -170 °C in a 40% (v/v) PEG 400 final concentration. As the crystals were grown in the presence of high NaCl concentrations, they had to be quickly transferred to solutions of diluted reservoir solution with successively higher concentrations of PEG 400. The final X-ray data set was collected on a single 0.25-mm crystal that had been soaked for 30 min each in solutions containing 30% of the reservoir solution and 10, 20, 30 and 40% (final concentrations) of PEG 400. Data were collected at the Cornell High Energy Synchrotron Source (CHESS) with 0.908 Å X-rays and recorded on Fuji image plates. For each image, the crystal was oscillated 0.3° for 30–40 s at a crystal-to-film distance of 400 mm. Images (198) were processed using DENZO<sup>24</sup> and the integrated intensities scaled together using SCALEPACK<sup>24</sup> using I/σ cutoff of -0.5 (Table 1).

**Structure determination.** The crystals of the virus-Fab complex belong to the rhombohedral space group, R3 ( $a = 372$  Å,  $\alpha = 108.4$  Å).  $V_m$  calculations **author: define  $V_m$**  indicated the presence of only one virus particle, saturated with Fab fragments, per unit cell. The orientation of the particle was defined by strong 5-fold (~25σ), 3-fold (~23σ) and 2-fold (~20σ) peaks in the self-rotation function, calculated using the program GRFP<sup>25</sup>. The known structures of Fab17-IA (ref. 26), HRV-14 (ref. 3), and the Fab17-IA/HRV14 cryo-EM image reconstruction<sup>5</sup>, were used to place an entire HRV-14-(Fab)<sub>60</sub> particle in the rhombohedral cell at (0,0,0) in the orientation defined by the self-rotation function calculation. Structure-factor amplitudes and phases were calculated from the Cα coordinates of the model to 8 Å using the program SFALL from the CCP4 package and used to create a molecular envelope mask for 20-fold averaging using Rossmann's suite of programs<sup>27</sup>. Phases were extended in steps of one reciprocal lattice point, and each step was followed by at least six cycles of 20-fold non-crystallographic averaging. Subsequent molecular masks were generated from the averaged electron density with an assumed protein content of 53% in the crystal. A total of eight different masks were used during the 47 steps of phase extensions and 300 cycles of averaging that were required to attain an initial density map at 4 Å resolution. This 4 Å map clearly showed that the initial position of the Fab model was up to 4 Å away from the correct position (Fig. 2); the conformation of the CDR3 loop of the heavy chain differed from that in the unliganded structure; the HRV-14 density was consistent with the initial model; and the electron density for the Fab constant domains was diffuse and uninterpretable. Therefore, the constant domains were removed from the model. The electron density was 20-fold-averaged for ~15 cycles during each of the three cycles of phase refinement. This model, without the constant domains, was then refined using X-PLOR<sup>28</sup>. An overall  $B$  value of 20 Å<sup>2</sup> and all data were used for Powell positional refinement. The initial  $R$  factor for the model was 29.7% using all data between 10.0 and 4.0 Å resolution, and the  $R$  factor of the current model is 21.2%, with geometrical statistics typical for a structure determined to >3.5 Å resolution. Buried surface areas were calculated using

TABLE 1 Distribution and  $R_{\text{sym}}$  of the HRV-14/Fab17-IA complex data

| Resolution (Å) | $R$ -factor (%) | % Total data |
|----------------|-----------------|--------------|
| 20.00–7.86     | 6.3             | 79.1         |
| 7.86–6.30      | 13.8            | 72.6         |
| 6.30–5.52      | 18.6            | 68.1         |
| 5.52–5.03      | 19.2            | 67.5         |
| 5.03–4.67      | 19.5            | 66.1         |
| 4.67–4.40      | 22.1            | 61.8         |
| 4.40–4.18      | 25.1            | 53.6         |
| 4.18–4.00      | 29.3            | 46.9         |
| Total          | 14.9            | 64.5         |



a 1.7 Å probe radius<sup>29</sup>. Atomic coordinates for this model have been submitted to the Brookhaven Protein Database for distribution.

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1. Mosser, A. G., Leippe, D. M. & Rueckert, R. R. in *Molecular Aspects of Picornavirus Infection and Detection* (eds Semler, B. L. & Ehrenfeld, E.) 155–167 (Am. Soc. Microbiol., Washington, DC, 1989).
2. Smith, T. J., Olson, N. H., Cheng, R. H., Chase, E. S. & Baker, T. S. *Proc. Natl Acad. Sci. USA* **90**, 7015–7018 (1993).
3. Rossmann, M. G. *et al. Nature* **317**, 145–153 (1985).
4. Olson, N. H. *et al. Proc. Natl Acad. Sci. USA* **90**, 507–511 (1993).
5. Smith, T. J. *et al. J. Virol.* **67**, 1148–1158 (1993).
6. Kabat, E. A., Wu, T. T., Reid-Miller, M., Perry, H. M. & Gottesman, K. S. (US Department of Health and Human Services, Public Health Service, NIH, Bethesda, MD, 1987).
7. Tomlinson, I. M. *et al. J. Mol. Biol.* **256**, 813–817 (1996).
8. Stanfield, R. L. & Wilson, I. A. *Trends Biotechnol.* **12**, 275–279 (1994).
9. Smith, T. J. *et al. Science* **233**, 1286–1293 (1986).
10. Oliveira, M. A. *et al. Structure* **1**, 51–68 (1993).
11. Rossmann, M. G. *J. Biol. Chem.* **264**, 14587–14590 (1989).
12. Colonna, R. J., Callahan, P. L., Leippe, D. M. & Rueckert, R. R. *J. Virol.* **63**, 36–42 (1989).
13. Lee, W.-M. thesis, Univ. Wisconsin, USA (1991).
14. Wien, M. W. *et al. Nature Struct. Biol.* **2**, 232–243 (1995).
15. McCullough, K. C. *et al. J. Virol.* **66**, 1835–1840 (1992).
16. Acharya, R. *et al. Nature* **327**, 709–716 (1989).
17. Verduguer, N. *et al. EMBO J.* **14**, 1690–1696 (1995).
18. Wang, K.-S., Schmaljohn, A. L., Kuhn, R. J. & Strauss, J. H. *Virology* **181**, 694–702 (1991).
19. Smith, T. J. *et al. Proc. Natl Acad. Sci. USA* **92**, 10648–10652 (1995).
20. Harber, J., Bernhardt, G., Lu, H.-H., Sgro, J.-Y. & Wimmer, E. *Virology* **214**, 559–570 (1995).
21. Bizebard, T. *et al. Nature* **376**, 92–94 (1995).
22. Hoover-Litty, H. & Greve, J. M. *J. Virol.* **67**, 390–397 (1993).
23. Arnold, E. *et al. J. Mol. Biol.* **177**, 417–430 (1984).
24. Otwinowski, Z. in *Data Collection and Processing* (eds Sawyer, L., Isaacs, N. & Bailey, S.) 56–62 (SERC Daresbury Laboratory, Warrington, UK, 1993).
25. Tong, L. & Rossmann, M. G. *Acta Crystallogr. A* **46**, 783–792 (1990).
26. Liu, H. *et al. J. Mol. Biol.* **240**, 127–137 (1994).
27. Rossmann, M. G. *et al. J. Appl. Cryst.* **25**, 166–180 (1992).
28. Brünger, A. T. *X-plor (Version 3.1) User's Guide* (Yale Univ., New Haven, CT, 1992).
29. Sheriff, S. *Immunomethods* **3**, 191–196 (1993).
30. Luo, M. *et al. Science* **235**, 182–191 (1987).

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## Control of telomere growth by interactions of RAP1 with the most distal telomeric repeats

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**TELOMERES**, the specialized DNA–protein structures at the ends of eukaryotic chromosomes, are required for chromosomal stability and integrity<sup>1–3</sup>. Regulation of the overall length of the telomeric DNA repeat tract is likely to be a key requirement for its various biological roles. We have studied telomere length regulation in the yeast *Cluyveromyces lactis*, which has long (25 base pairs) homogeneous telomeric repeat units<sup>4</sup> that make it highly suitable for telomere studies. In the related *Saccharomyces cerevisiae*, the DNA-sequence-specific duplex-binding protein RAP1 is a component of the telomeric complex<sup>5–9</sup>. Here we show that the phenotypic severity of previously described telomerase RNA (*ter1*) mutations<sup>10</sup> is directly proportional to the loss of RAP1 binding to mutated telomeric repeats. Using a carboxy-terminal-tail mutant of *K. lactis* RAP1, we also show that, unexpectedly, RAP1 interaction with the most terminal telomeric repeats is crucial for telomere length control.

We tested whether the phenotypes of five *K. lactis* *ter1* template mutations<sup>10</sup> (Fig. 1a) were caused by disruption of *K. lactis* RAP1

(KIRAP1) binding to telomeres. First we demonstrated that *K. lactis* contains binding activity *in vitro* for telomeric sequence duplex oligonucleotides with the properties expected for RAP1 protein, and then we identified this activity as KIRAP1. A specific complex was formed with *K. lactis* cell extract and a telomeric oligonucleotide (Fig. 1b, lane 1). Only an oligonucleotide permutation bearing an intact RAP1-binding site was able to compete for the shifted complex (Fig. 1b, oligonucleotide 14–13, lanes 5–7). Permutations that split this site (Fig. 1b, oligonucleotide 1–25, lanes 2–4, or oligonucleotide 22–21, lanes 8, 9 and 10) were unable to compete for binding. Single-stranded oligonucleotides corresponding to either strand were also unable to compete even when present in 3,000-fold excess (results not shown).

We next tested whether duplex oligonucleotides corresponding to the various *ter1* mutations (Fig. 1a) had altered RAP1 binding abilities in the gel-shift assay (Fig. 1c, d). The *ter1-Bgl* and *ter1-Kpn* mutations alter residues outside the RAP1 binding consensus and their only immediate effect *in vivo* is a slight shortening of the telomere<sup>10</sup>. The corresponding oligonucleotides showed efficient binding in both competition assays (Bgl, Fig. 1c, lanes 8–13; Kpn, Fig. 1c, lanes 14–19) and direct-binding assays (Fig. 1d, lanes 3, 4, 7 and 8). The *ter1-AA* mutation, which changes a pair of G residues to A at two purine positions in the RAP1 consensus sequence, causes gradual and mild telomere lengthening (ref. 10 and M. J. McEachern and E.H.B., unpublished observations). The corresponding (AA) oligonucleotide showed intermediate binding, both competing and binding approximately one-tenth as well as the wild-type oligonucleotide (Fig. 1c, lanes 21–26; Fig. 1d, lanes 11 and 12). The *ter1-BsiW* and *ter1-Acc* mutations are at conserved RAP1 consensus residues 4 and 5 respectively (Fig. 1a) and cause moderate (*ter1-BsiW*) or severe (*ter1-Acc*) immediate telomere lengthening *in vivo*<sup>10</sup>. Binding of the corresponding mutant oligonucleotides was greatly reduced compared with wild-type in both competition assays (Fig. 1c, Bsi, lanes 27–32; Acc, lanes 33–35), and direct-binding assays (Fig. 1d, Bsi, lanes 5 and 6; Acc, lanes 9 and 10). The competitive abilities of Bsi and Acc oligonucleotides were reduced 30- and 100-fold, respectively. In summary, binding affinities fell into the series  $\text{Acc} < \text{Bsi} < \text{AA} < \text{wild-type} \approx \text{Kpn} < \text{Bgl}$ . Hence, for each of these mutations, the degree of loss of binding ability *in vitro* was correlated directly with the severity of the immediate loss of telomere length regulation *in vivo*<sup>10</sup>.

To confirm that the binding activity in *K. lactis* extracts was indeed KIRAP1, we made extracts from *S. cerevisiae* cells expressing the cloned KIRAP1 gene. As shown in Fig. 2, lane 4, this extract shifted the *K. lactis* wild-type telomeric oligonucleotide into a major band with the same mobility as the extracts prepared from *K. lactis* cells (Fig. 2, lane 2, filled arrow). Furthermore, the various mutant oligonucleotides competed for this shifted complex in a manner identical to the way they competed for the complex from the *K. lactis* cell extract (Fig. 2, lanes 5–9). The endogenous ScRAP1 from control *S. cerevisiae* cells (open arrow), behaved similarly in competition experiments (Fig. 2, lanes 10–14). The difference in mobility of the full-sized shifted complexes produced by the *S. cerevisiae* and the *K. lactis* activities was consistent with the predicted size difference between the KIRAP1 and ScRAP1 proteins (666 and 827 amino acids respectively). We conclude that the *K. lactis* binding activity is the KIRAP1 protein.

A minimal ScRAP1 domain affecting telomere length has previously been identified at the C-terminal tail<sup>11</sup>. Interestingly, within this 25-residue C-terminal tail domain, each ScRAP1 residue critical for telomeric silencing and size control is conserved in the corresponding C-terminal tail of the predicted KIRAP1<sup>11,12</sup>. Replacing the wild-type *K. lactis* RAP1 gene with a *rap1* allele lacking the normal C-terminal tail (*rap1-ΔC*) resulted in a modest (~100 bp), stable increase in mean telomere size (Fig. 3a, lanes 3 and 5). This very small effect on telomere length suggested that any effect of this *rap1-ΔC* allele alone on telomerase activity must be minimal. The RAP1-ΔC protein had

DNA binding and competition properties *in vitro*, with both the wild-type and the various mutated oligonucleotides, which were indistinguishable from those of the wild-type protein (Fig. 3b, and results not shown).

To analyse the role of KIRAP1 in telomere length regulation, we combined the C-terminal mutation of KIRAP1 with either the *ter1-AA* or *ter1-BsiW* mutations. In the *ter1-AA;rap1-ΔC* double mutant, telomere length regulation was lost from a fraction of the telomeres within the first passage (~25 cell divisions), with the telomeric restriction fragment profile consisting of both the discrete *rap1-ΔC* pattern (compare lanes 1 and 3 with lane 5) and a superimposed smear of fragments. Comparison of differential hybridizations with an AA-specific probe and a wild-type probe showed that, at this point, the discrete fragments contained an average of no more than one or two AA repeats (results not shown), consistent with previous results in a wild-type RAP1 background<sup>10</sup>. Therefore the immediate loss of telomere lengths control on creation of the double mutant occurred on telomeres that contained very few terminal AA repeats. Loss of control was progressive, as by the third passage the pattern consisted almost completely of a smear extending to high-molecular-mass regions of the gel, indicating that most telomeres had lost length control (Fig. 3c, lanes 2 and 4).

When the *rap1-ΔC* mutation was combined with the more severe *ter1-BsiW* mutation, the telomeres were markedly increased

in size and length heterogeneity as soon as they could be analysed after replacement of the wild-type *TER1* gene (Fig. 3d, lanes 1–5). In contrast, in the single mutant *ter1-BsiW*, telomeres were only a few kilobases longer than in the wild type (Fig. 3d, lanes 8 and 9) and never reached the sizes seen in the double mutant, even after prolonged passaging<sup>10</sup>. Furthermore, the double mutant *ter1-BsiW;rap1-ΔC* had a markedly decreased cell viability (results not shown). Hence there was a strong synthetic lethal interaction between the *ter1* and the *rap1-ΔC* mutations, each of which by itself caused only mild effects on telomeres and no loss of viability.

As with the *ter1-AA;rap1-ΔC* strains, the extreme loss of telomere-length regulation in the double mutant *ter1-BsiW;rap1-ΔC* was not attributable to loss of its centromere-proximal tracts of wild-type repeats, as these were intact (and even slightly longer than in the *ter1-BsiW* single mutant). This was determined by double digestion with *EcoRI* and *BsiWI*, which cuts only the mutant telomeric repeats (Fig. 3d: compare lanes 10 and 12).

Therefore the RAP1-ΔC protein was incapable of mediating telomere length control once the most terminal wild-type repeats were replaced by AA or BsiW repeats. This suggested that a primary mode of telomere length regulation involves KIRAP1 and, surprisingly, the duplex repeat sequences at the very end of the telomere.

To test this hypothesis directly, we reintroduced a wild-type *TER1* gene into a *ter1-AA;rap1-ΔC* double mutant and analysed

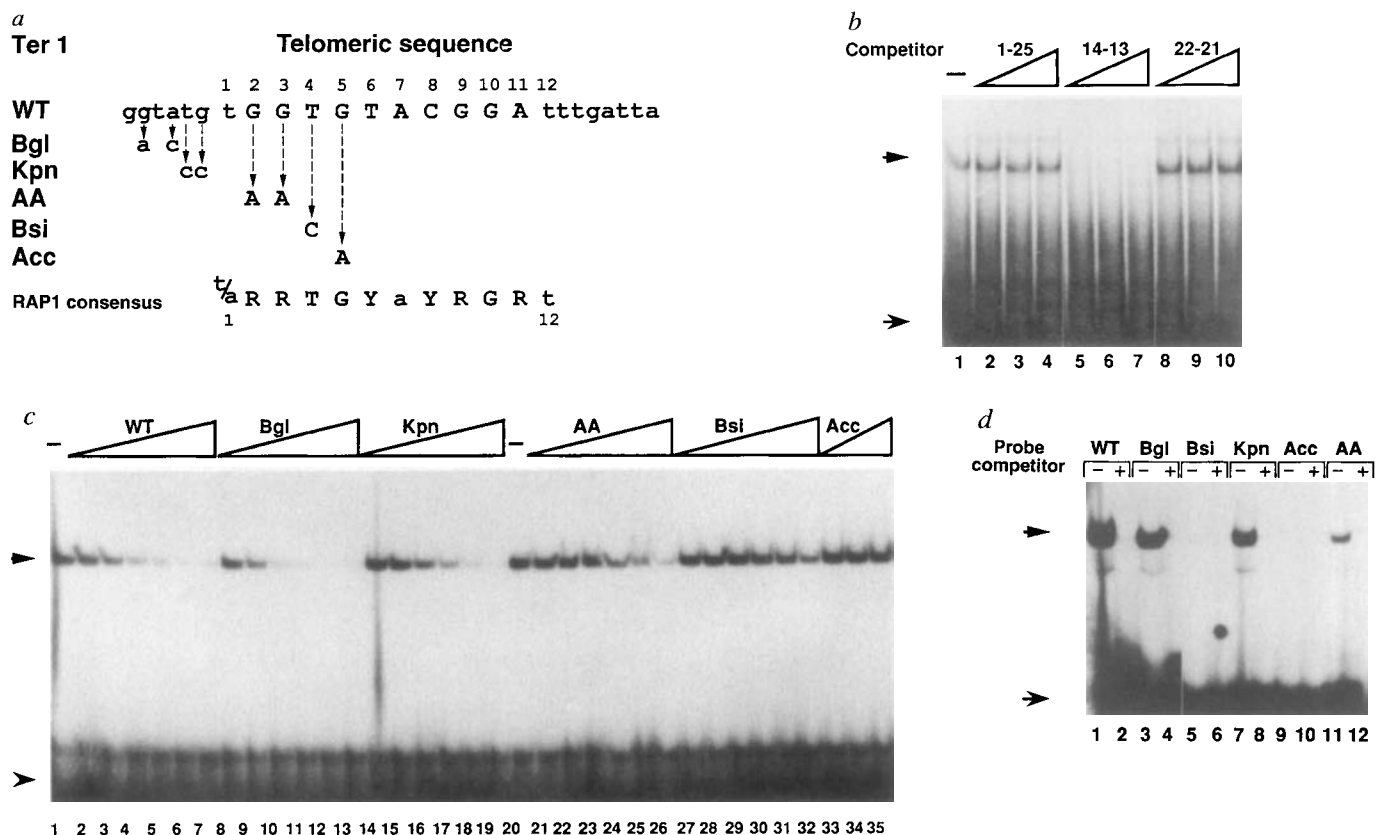


FIG. 1 The RAP1 binding consensus is disrupted in telomere-lengthening *ter1* mutants. *a*, Telomeric sequences of *ter1* mutant repeats. Mutations are indicated by arrows; upper case indicates highly conserved bases; numbers indicate alignment to RAP1 consensus. *b*, A gel-shift assay with telomeric double-stranded 60-mer end-labelled probe with one complete RAP1 binding and extract prepared from *K. lactis* strain K7B520. Lane 1, no competitor; lanes 2–4, permutation 1–25 at 3×, 10× and 30× molar excess, respectively. Lanes 5–7, permutation 14–13 with 3×, 10× and 30× molar excess, respectively; and lanes 8–10, permutation 22–21 with 3×, 10× and 30× molar excess, respectively. Free DNA is indicated as open arrow and shifted complex as closed arrow. *c*, Gel-shift assay with

double-stranded end-labelled 14–13 oligonucleotide as probe with the different double-stranded 25-mer oligonucleotide shown in *a* as competitors. Lane 1, no competitor; lanes 2–7, wild type (WT); lanes 8–13, Bgl; lanes 14–19, Kpn; lanes 21–26, AA; lanes 27–32, Bsi; with 3, 10, 30, 100, 300, 1,000× molar excess, respectively; and lanes 33–35, Acc with 100, 300, 1,000× molar excess, respectively. *d*, a Gel-shift assay with the different mutant oligonucleotides as probes, in the presence or absence of 30× molar excess wild-type (WT) competitor. Lanes 1 and 2, wild type; lanes 3 and 4, Bgl; lanes 5 and 6, Bsi; lanes 7 and 8, Kpn; lanes 9 and 10, Acc; lanes 11 and 12, AA.

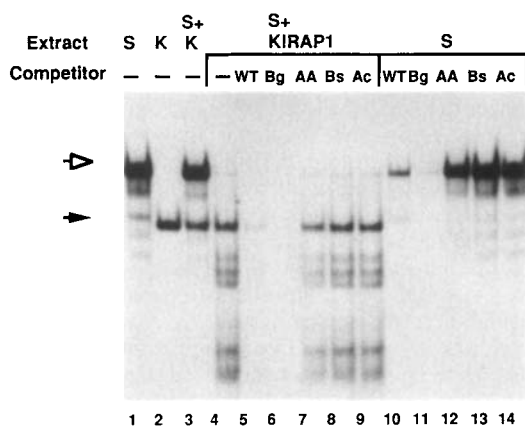


FIG. 2 Extracts from *S. cerevisiae* cells expressing *KIRAP1* behave identically in gel-shift assays to the *K. lactis* cell extract activity. Extracts were used to shift oligonucleotide 14-13. Lane 1 and lanes 10–14, *S. cerevisiae* extract (S); lane 2, *K. lactis* extract (K); lanes 4–9, extracts from *S. cerevisiae* cells expressing *KIRAP1* from its own promoter (S+*KIRAP1*). Double-stranded competitors (30× molar excess) were added as indicated. Open arrowhead, *S. cerevisiae* RAP1 complex; closed arrowhead, *K. lactis* RAP1 complex. Shifted bands below the major band represent *KIRAP1* degradation products, as they are dependent on the presence of the *KIRAP1* construct and have identical competition properties to the full-length band. Lane 3, equal amounts of *S. cerevisiae* and *K. lactis* extract mixed (S+K), indicating that the reduction in ScRAP1 shifted complex in extracts expressing *KIRAP1* (lane 4–9) was not due to competition. Protein gels showed reduced ScRAP1 in *S. cerevisiae* cells containing the *K. lactis* RAP1 gene (data not shown).

telomeric lengths. Immediately after introduction of the wild-type *TER1* gene, a discrete telomeric banding pattern appeared in all clones tested (Fig. 3e, lanes 1–5, and results not shown), whereas the double mutant retained its smear of drastically elongated telomeres lacking any resemblance to the wild-type pattern (Fig. 3e, lane 6). The smear of fragments included the size range of discrete fragments that appeared after the wild-type telomerase RNA gene was reintroduced. When the wild-type *TER1* was reintroduced after the fourth passaging of the *ter1-AA;rap1-ΔC* strain, again the telomeres 'froze' in a discrete pattern, but now with longer telomeres (results not shown).

We propose that in *K. lactis*, as in *S. cerevisiae*<sup>13–15</sup>, RAP1 bound to duplex telomeric DNA is involved in forming a structured, higher-order chromatin complex at telomeres. This may involve the products of the *SIR* genes, which in *S. cerevisiae* have been shown to interact with the C-terminal domain of chromosomally bound ScRAP1 to set up a silent chromatin domain<sup>16,17</sup>. Consistent with this possibility, combining the *ter1-BsiW* mutation with a *K. lactis sir2* deletion<sup>18</sup> resulted in a synergistic loss of telomere length regulation very similar to that seen with *KIRAP1-ΔC* (results not shown). Therefore we propose a model (Fig. 4), in which telomere length control is predominantly exerted by sequence-specific

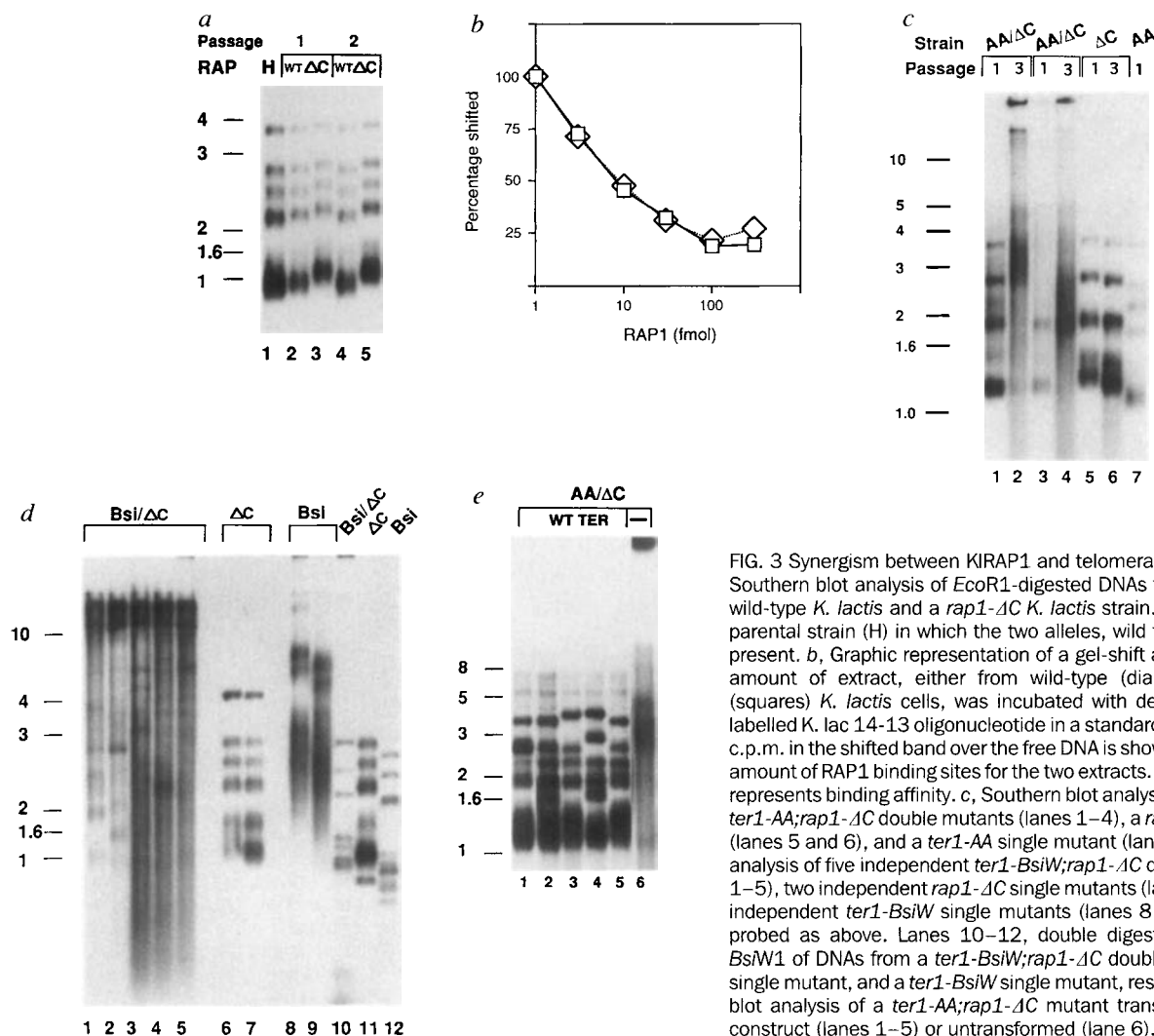


FIG. 3 Synergism between *KIRAP1* and telomerase RNA mutations. **a**, Southern blot analysis of *EcoR1*-digested DNAs from two passages of wild-type *K. lactis* and a *rap1-ΔC* *K. lactis* strain. Lane 1, heteroallelic parental strain (H) in which the two alleles, wild type (WT) and ΔC are present. **b**, Graphic representation of a gel-shift assay in which a fixed amount of extract, either from wild-type (diamonds) or *rap1-ΔC* (squares) *K. lactis* cells, was incubated with decreasing amounts of labelled *K. lac* 14-13 oligonucleotide in a standard reaction. The ratio of c.p.m. in the shifted band over the free DNA is shown as a function of the amount of RAP1 binding sites for the two extracts. The slope of the curve represents binding affinity. **c**, Southern blot analysis of two independent *ter1-AA;rap1-ΔC* double mutants (lanes 1–4), a *rap1-ΔC* single mutant (lanes 5 and 6), and a *ter1-AA* single mutant (lane 7). **d**, Southern blot analysis of five independent *ter1-BsiW;rap1-ΔC* double mutants (lanes 1–5), two independent *rap1-ΔC* single mutants (lanes 6 and 7) and two independent *ter1-BsiW* single mutants (lanes 8 and 9) digested and probed as above. Lanes 10–12, double digestion with *EcoR1* and *BsiW1* of DNAs from a *ter1-BsiW;rap1-ΔC* double mutant, a *rap1-ΔC* single mutant, and a *ter1-BsiW* single mutant, respectively. **e**, Southern blot analysis of a *ter1-AA;rap1-ΔC* mutant transformed with a *TER1* construct (lanes 1–5) or untransformed (lane 6).





*cerevisiae*. Mutations that were lethal in combination with a null allele of the gene encoding the nucleoporin Nup100 (ref. 8) were identified by a colony sectoring assay (R.M., J. Watkins, S.R.W., manuscript submitted). The mutants were classified into three new complementation groups, and the *gle1* (for GLFG (glycine-leucine-phenylalanine-glycine) lethal) mutants analysed. The *gle1* mutants were also lethal in combination with a null allele of *nup116* (ref. 8), but not with a *nup145* amino-terminal-deletion mutant<sup>9</sup> (data not shown).

Five temperature-sensitive *gle1* alleles were isolated and backcrossed to a wild-type *NUP100* background for measurement of nuclear transport capacity. Their phenotypes were ranked in relative order of increasing severity and penetrance, and all behaved in the same way apart from the strength of their defects. After the shift to the non-permissive growth temperature, the *gle1-4* mutant showed rapid inhibition of poly(A)<sup>+</sup> RNA export (within 10 min) (Fig. 1a). Nucleolar fragmentation has been observed in several nuclear transport mutants<sup>10</sup>. In temperature-arrested *gle1-4* cells, the nucleolar protein Nop1 remained in the nucleus but fragmented from a single intranuclear crescent to multiple discrete nuclear foci (Fig. 1b). Over the same time frame, *gle1-4* cells were competent for nuclear protein import (assayed as in ref. 11; data not shown). Thin-section electron microscopic analysis revealed that the nuclear pore complex and nuclear envelope structure were intact (Fig. 1c, d), indicating a specific requirement for *GLE1* in mediating RNA export.

The wild-type *GLE1* gene was cloned by complementation of the *gle1-1* non-sectoring and temperature-sensitive phenotype with a yeast genomic library. The 2.2-kilobase(kb) minimal complementing DNA fragment contained a single 1,614-base-pair(bp) open reading frame. To test whether *GLE1* encodes an essential gene product, we replaced one copy of the gene with *HIS3* in a diploid strain. After sporulation and dissection, only two viable His<sup>-</sup> haploid spores were recovered per tetrad (data not shown). Growth of the *gle1* null spores arrested after 1 to 5 cell divisions.

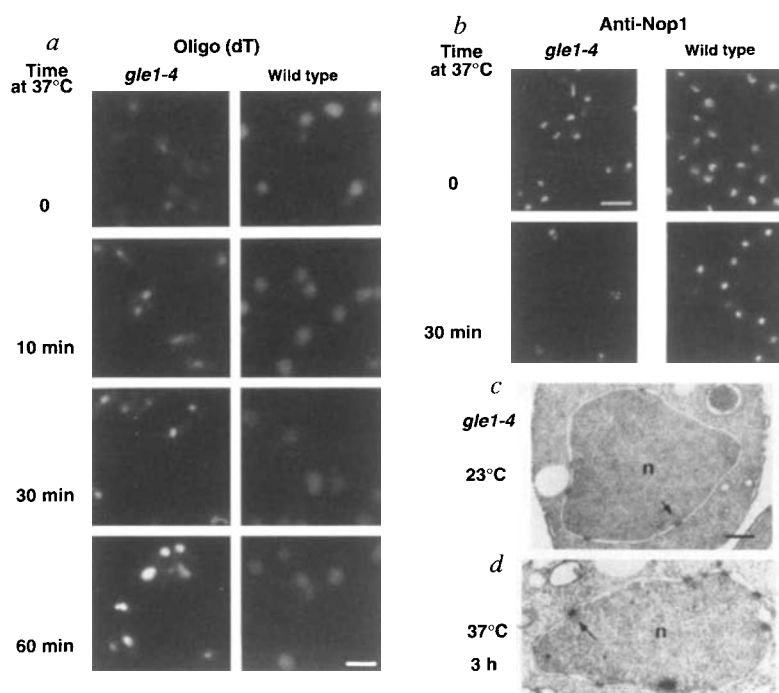
The predicted amino-acid sequence for Gle1 (M<sub>r</sub> 62K) is shown in Fig. 2a. No significant sequence similarities to any other proteins in the databases were detected using FASTA/BLAST programs<sup>12</sup>. The middle region of Gle1 is enriched in charged residues, and there is a leucine-rich peptide octamer with homology to the NESs in Rev<sup>2,3</sup>, the heat-stable protein kinase inhibitor

(PKI)<sup>2</sup>, and TFIIA<sup>13</sup> (Fig. 2b). To test whether the putative NES in Gle1 could mediate nuclear export, a peptide corresponding to amino acids 350–358 of Gle1 was conjugated through an N-terminal cysteine residue to human serum albumin (HSA). The HSA–Gle1–NES conjugate and HSA alone were each labelled with different fluorescent dyes and co-injected into the nuclei of COS-1 cells. After 30 min at 37°C, HSA–Gle1–NES was exported to the cytoplasm, whereas the HSA alone remained in the nucleus (Fig. 2c). Therefore, Gle1 has a bona fide NES which is sufficient to mediate nuclear export.

To analyse whether the NES was necessary for Gle1 function, an in-frame deletion spanning amino acids 351–358 was made ( $\Delta$ NES). When *gle1* $\Delta$ NES replaced wild-type *GLE1*, the cells were inviable at all growth temperatures (data not shown), indicating that the lack of the NES results in complete loss of function. For the NESs of Rev<sup>14</sup>, PKI<sup>2</sup> and TFIIA<sup>13</sup>, single mutation to alanine of the residues underlined in Fig. 2b blocks nuclear export. We replaced each of the four leucine residues in Gle1 at amino-acid positions 351, 353, 356 and 358 individually with alanine, and assayed for cell growth and RNA-export capacity. Although they differed in the degree of severity, all four mutations showed perturbations. Cells with the L356A mutation were temperature-sensitive, whereas cells with the other three mutations were viable at 37°C (data not shown). At 37°C, poly(A)<sup>+</sup> RNA export was inhibited in >95% of the *gle1-L356A* cells, and in at least 20% of *gle1-L351A*, *gle1-L353A* and *gle1-L358A* cells (Fig. 2d). Under identical conditions, RNA export was not altered in wild-type cells. To test whether the *in vivo* phenotypes of the mutations correlated with the nuclear export activity of the NES motif, corresponding mutated peptides were analysed by the microinjection assay. The mutated peptides were not exported, indicating that the export activity of the NES in Gle1 is probably required to maintain wild-type levels of RNA export *in vivo*.

Rip1 from yeast<sup>1</sup> and human Rip1/Rab<sup>4,5</sup> are nuclear pore complex and/or nuclear proteins that interact with the NES domain of Rev in the yeast two-hybrid assay. This region of Rev also interacts with the nucleoporin Nup100 (ref. 1), and we identified *GLE1* in a genetic screen with a *nup100* mutant. To determine whether Gle1 interacts with yeast Rip1 and/or Nup100, a yeast two-hybrid assay was done. The C-terminal portion of Gle1

FIG. 1 Analysis of *gle1-4* nuclear transport capacity. **a**, Nuclear export of poly(A)<sup>+</sup> RNA is blocked in *gle1-4* cells. *In situ* hybridization with a digoxigenin-coupled oligo(dT) probe was conducted with *gle1-4* and wild type cells after growth at 37°C for the designated times. Probe localization was detected with an FITC-coupled anti-digoxigenin antibody. Compared with the diffuse cytoplasmic staining in the wild-type cells, marked nuclear accumulation in the *gle1-4* cells is apparent at 10 min, and increases with time. Scale bar, 5  $\mu$ m. **b**, Nucleolar structure in the *gle1-4* cells fragmented into multiple foci in a temperature-dependent manner, as assayed by indirect immunofluorescence with the monoclonal antibody D77 against Nop1<sup>25</sup>. Nucleolar staining in wild-type cells remains a single crescent-shaped structure. Scale bar, 5  $\mu$ m. **c**, Thin-section electron micrographs of *gle1-4* cells at 23°C, and **d**, at 37°C for 3 h; n, nucleus; arrow, NPCs; scale bar, 0.25  $\mu$ m.



interacted strongly with Rip1 and weakly with the N-terminal GLFG-repetitive region of Nup100 (Fig. 3a). The  $\Delta$ NES mutation abolished interaction with Rip1, and the L356A mutation decreased activation by at least twofold (Fig. 3a). Thus the Gle1 NES domain is required for interaction with Rip1. The relative decrease in Rip1 interaction was the same when wild type Rev and

the analogous L81A mutation in Rev were compared (Fig. 3a). A direct protein-protein interaction between Gle1 and Rip1 was confirmed in a ligand blot-overlay assay with full-length, bacterially expressed proteins (Fig. 3b).

To analyse Gle1 further, the subcellular localization of epitope-tagged Gle1 was determined by indirect immunofluorescence microscopy. Five tandem IgG binding domains from *Staphylococcus aureus* protein A were fused to the Gle1 N terminus, and the epitope-tagged gene sustained wild-type growth in a *gle1*-null background. Protein A-Gle1 staining was concentrated in a punctate pattern at the nuclear periphery (Fig. 4) and was typical of yeast nuclear pore complexes<sup>8</sup>. Therefore, Gle1 may be a structural component of the nuclear pore complex; alternatively, Gle1 may merely be concentrated at its site of action, just as soluble nuclear import factors are preferentially localized at the nuclear pore and the nuclear periphery<sup>15-18</sup>.

**a**

```

MEFVDFEVFNSDTDSPEEETCSTTSSTSSQCPTPEPSPAIKLPSTFKVGTGKLVNESVV 60
ILDPALENALRDNLQSKLIPINEPIVAASSIIVPHSTNMPLFRASHSSLLDNAKNSAT 120
APLLEAIEESFORKMQNLVLNLANKEIQSIRENKRVEEQRKKEEERKRKEAEKAKRE 180
QELLRQKKDEEERKRKEAEKLAQKQOEERKKIEEONEKEROLKKEHEAKLLQOKDKLG 240
KAVTNFDKISKMFWHYKDKIAQIKQDIVLPKKAQDVNVRNLLSRHKKINPKFGQLTNSN 300
QQLFKIQNELTQLINDTKGDSLAYHWILNPIAKAVVHQAEDEVVVKPESALPLGKLTLL 360
LVQFPPLQELFMARLVKKCPFVIGFTCEIDTEKGRQNMGWKRNNENKWEEDNTSYDERMGG 420
ILSLFAIITRLQLPQEFITTTSHPPIALSWHILARICNTPLNLITNTHFVLGSWDA 480
AVQPLQAYGNQASKLLILIGEELTSRMAEKKYVGAARLRILLEAWQNNNMESFPMS 538

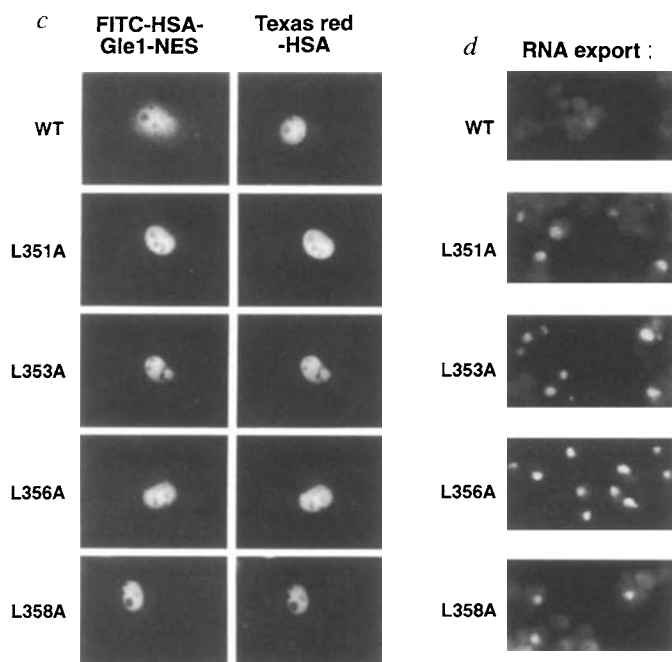
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**b**

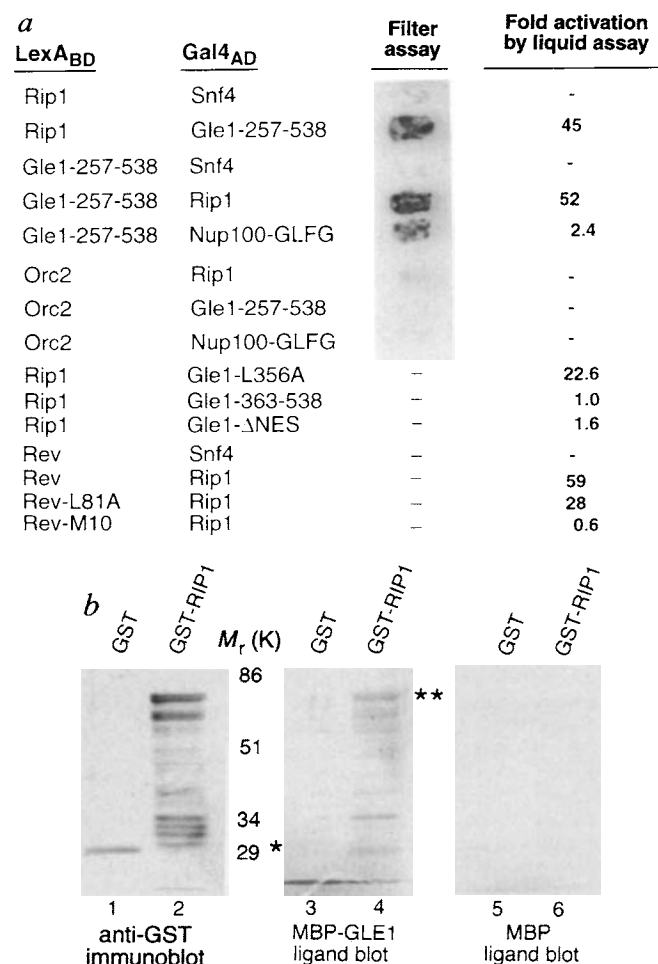
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aa      aa
Gle1    351  L P L G K L T L  358
Rev     75  L P P L E R L T L  83
PKI     37  L A L K L A G L D I  46
TFIIIA  L P V L E N L T L K  C terminus

```



**FIG. 2** *Gle1* is a unique and essential gene that requires a NES for poly(A)<sup>+</sup> RNA export. **a**, *Gle1* amino-acid sequence. Charged amino acids are underlined and an NES-like sequence is boxed. **b**, Alignment of a region of *Gle1* with the NES sequences of HIV-1 Rev<sup>2,3</sup>, PKI<sup>2</sup> and TFIIIA<sup>43</sup>. The underlined residues are required for NES function, as tested by single mutation to alanine<sup>2,13,14</sup>. **c**, The NES motif in *Gle1* can mediate the nuclear export of HSA in microinjected COS-1 tissue culture cells. The fluorescent FITC staining in the cytoplasm reflects export of the respective NES peptide conjugate, FITC staining in the nucleus reflects the lack of export and, as a control, Texas red staining from co-injected unconjugated HSA remains concentrated in the nucleus. **d**, Export of poly(A)<sup>+</sup> RNA is inhibited by mutations in the NES of *Gle1*. Nuclear accumulation of poly(A)<sup>+</sup> RNA was detected by *in situ* hybridization after growth at 37 °C for either 5 (L356A) or 16 h (WT, L351A, L353A, L358A).



**FIG. 3** Characterization of *Gle1* protein-protein interactions. **a**, Interaction of *Gle1* with Rip1 and Nup100 in the yeast two-hybrid system. Both filter and liquid  $\beta$ -galactosidase assays were done<sup>28</sup>. Plasmids expressing Gal4<sub>AD</sub>-Snf4 (ref. 29) and LexA<sub>BD</sub>-Orc2 (ref. 28) confirmed the specificity of the interactions. Full-length *Gle1* fused to Gal4<sub>AD</sub> interacted with Rip1 at approximately the same level as *Gle1*-257-538 (data not shown). **b**, *In vitro* *Gle1* and Rip1 interactions. Bacterial lysates from strains expressing either GST, alone or GST-Rip1 were separated on 9% SDS-polyacrylamide gels and transferred to nitrocellulose. Identical blots were analysed either by immunoblotting with a polyclonal antibody against GST (left) or by ligand blot overlay with purified MBP-Gle1 (middle) or purified MBP (right) and polyclonal antibody against MBP (middle and right). The MBP-Gle1 specifically bound to GST-Rip1 (\*\*) and its proteolytic products (lane 4), but not to GST alone (\*) (lane 3). The specificity overlay with MBP alone is blank (lanes 5 and 6).

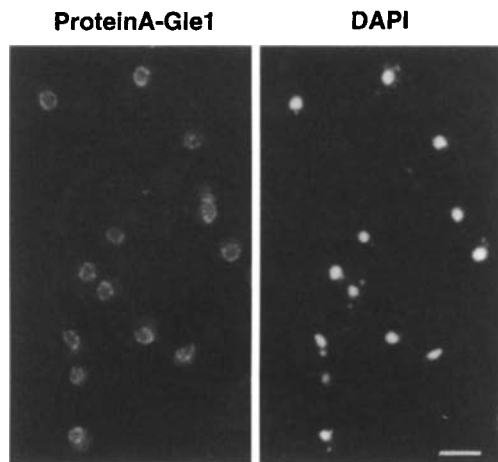


FIG. 4 Intracellular distribution of Gle1 was determined by indirect immunofluorescence microscopy with a protein A–Gle1 fusion protein. Cells were fixed for 5 min and probed with a rabbit anti-mouse antibody (Cappel Labs; 1:200 dilution) and with a Texas-red-conjugated goat anti-rabbit antibody (Cappel Labs; 1:200). Coincident staining of the nuclei with diaminophenylindole (DAPI) is shown.

Our results suggest that Gle1 has a direct and essential role in the nuclear export of mRNAs. To our knowledge, this is the first example of a cellular protein with an NES whose mutation blocks RNA export. Our results also highlight features common to Gle1 and Rev; both require an NES to mediate RNA export and both interact with Rip1 and Nup100. This reinforces the premise that the export pathways for viral RNAs and cellular mRNAs are overlapping. The NES domain of Rev is C-terminal to a domain required for RNA binding, oligomerization and nuclear localization<sup>19–24</sup>. The precise role for Gle1 in RNA export may be revealed by analysing its unique N-terminal region. The mechanism of Gle1 action may involve the direct targeting of RNA/RNP molecules to or through the nuclear pore complex by means of Rip1 and Nup100 interactions. □

## Methods

**Microscopy.** Thin-section electron microscopy, immunofluorescence microscopy and *in situ* hybridization were done as described<sup>26</sup>.

**Microinjection assay.** Wild-type Gle1–NES (amino-acid sequence CALPLGKLT) and the L356A mutated peptides were synthesized on an ABI Model 431A machine. The L351A, L353A and L358A mutated peptides were synthesized by Research Genetics. 5–10 peptides were coupled to each HSA as described<sup>3</sup> and analysed by SDS–PAGE. Using FluoReporter protein-labelling kits (Molecular Probes), HSA–Gle1–NES conjugates were labelled with fluorescein isothiocyanate (FITC) and HSA was labelled with Texas red. COS-1 (African green-monkey kidney) cells were grown on coverslips in DMEM medium with 0.05 mM  $\beta$ -mercaptoethanol and 10% calf serum. Microinjection was achieved using a Narashigi micromanipulator and a constant-flow pipette. Cell nuclei were microinjected with a 1:1 mixture of the respective FITC–HSA–Gle1–NES and Texas red–HSA (protein concentration  $\sim 3$  mg ml<sup>-1</sup>). After 30 min at 37 °C, microinjected cells were fixed for 15 min in 3% formaldehyde/PBS, washed in PBS, and mounted with 90% glycerol, 1 mg ml<sup>-1</sup> *p*-phenylenediamine. Photographs were taken with the 40 $\times$  objective on an Olympus microscope with Kodak T-MAX 400 film.

**Construction of *gle* mutations.** The *gle1*-null strain was made by the method of Baudin<sup>27</sup> with pBM2815 and two 64-mer oligonucleotides: 6HISA, TCGCGGGCCGTTGCTACAGGATATTCGAGAGTCGAAGAATGAGGGCCCTCTAGTACACTC; 7HISA, TGTTATTAAATTTAGTGGCGTATTACATATTCAGCAAAATATGGCGCGCCTCGTTGAGAATG. This generated a  $\sim 1,100$ -bp *HIS3* fragment flanked at the 5' end by 45 bp of sequence from bp +5 to -40 of *GLE1* and at the 3' end by 45 bp from +1309 to +1354. The fragment was transformed into the diploid W303 (ref. 8), and *GLE1/gle1::HIS3* genotypes were confirmed by genomic colony polymerase chain reaction and by complementation with a *GLE1/LEU2/CEN* plasmid. For the protein A–Gle1 localization experiments, the *gle1*-null cells expressed an N-terminal fusion of five tandem IgG binding domains from *S. aureus* protein A to Gle1 (on a *TRP/CEN* plasmid and under the control of the

*NUP116* promoter<sup>8</sup>). Site-directed mutagenesis was used to delete the NES motif and change the leucine residues at positions 351, 353, 356 and 358 in Gle1 individually to alanine. The site of the mutations was confirmed by DNA sequence analysis. For phenotype analysis, the mutations were cloned in place of wild-type *GLE1* sequence into the yeast shuttle vector pRS315 (*LEU2 CEN*). A haploid *gle1*-null strain harbouring a *GLE1/URA3/CEN* plasmid was transformed with a *LEU/CEN* plasmid alone or one bearing either *GLE1*, *gle1*-L351A, *gle1*-L353A, *gle1*-L356A, or *gle1*-L358A. Growth was assayed by streaking the strains at 37 °C on synthetic complete/2% glucose medium containing 5-fluoro-orotic acid. Only strains that grow without the wild-type *GLE1/URA3* plasmid could form colonies, which were then tested for temperature-dependent growth.

**Two-hybrid assays.** Full-length Gle1, the Gle1– $\Delta$ NES, the C-terminal regions of Gle1 (amino acids 257–538, and 363–538), the C-terminal region of Gle1 (256–538) with the L356A mutation, the GLFG region of Nup100 (amino acids 2–595; ref. 8), and full-length Rip1 (ref. 1) were fused to the DNA-binding region of LexA (LexA<sub>BD</sub>) in the vector pCH436 and/or to the activation region of Gal4 (Gal4<sub>AD</sub>) in vector pCH358 (ref. 28). Wild-type Rev sequence was isolated from the LexA–Rev plasmid of Stutz *et al.*<sup>1</sup>. The L81A mutation in Rev was generated by site-directed mutagenesis, and both the mutated and wild-type Rev were fused to LexA<sub>BD</sub> in pCH436 (ref. 28). After co-transformation into yeast strain L40,  $\beta$ -galactosidase activity was assayed in triplicate by liquid assay<sup>26</sup> and by a filter assay<sup>28</sup>.

**Ligand-blot assay.** Full-length Gle1 was fused to maltose-binding protein (MBP) in pMAL-cRI (New England Biolabs), and full-length Rip1 was fused to glutathione-S-transferase (GST) in pGEX-3X (Pharmacia). Fusion proteins were expressed by induction with isopropyl- $\beta$ -D-thiogalactoside (IPTG) in DH5 $\alpha$  cells. MBP–Gle1 and MBP were purified on amylose resin according to manufacturer's instructions (New England Biolabs) and total cell extracts of GST–Rip1 and GST were prepared<sup>26</sup>. For ligand blot-overlay assays, blots were incubated as described<sup>26</sup>, using purified MBP–Gle1 or MBP at 0.1 mg ml<sup>-1</sup> in 20 mM HEPES–KOH (pH 7.3), 110 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA, 10 mM  $\beta$ -mercaptoethanol, 2 mg ml<sup>-1</sup> BSA. Binding of MBP–Gle1 or MBP was detected by incubating with a polyclonal antibody against MBP. Both the immunoblot and the ligand blot overlays were developed by incubation with affinity-purified, alkaline-phosphatase-conjugated anti-rabbit IgG and visualized with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-1-phosphate (Promega)<sup>26</sup>.

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- Stutz, F., Neville, M. & Rosbash, M. *Cell* **82**, 495–506 (1995).
- Wen, W., Meinkoth, J. L., Tsien, R. Y. & Taylor, S. S. *Cell* **82**, 463–473 (1995).
- Fischer, U., Huber, J., Boelens, W. C., Mattaj, I. W. & Lührmann, R. *Cell* **82**, 475–483 (1995).
- Bogerd, H. P., Fridell, R. A., Madore, S. & Cullen, B. R. *Cell* **82**, 485–494 (1995).
- Fritz, C. C., Zapp, M. L. & Green, M. R. *Nature* **376**, 530–533 (1995).
- Gerace, L. *Cell* **82**, 341–344 (1995).
- Gorlich, D. & Mattaj, I. W. *Science* **271**, 1513–1518 (1996).
- Wente, S. R., Rout, M. P. & Blobel, G. *J. Cell Biol.* **119**, 705–723 (1992).
- Wente, S. R. & Blobel, G. *J. Cell Biol.* **125**, 955–969 (1994).
- Schneider, R., Kadowaki, T. & Tartakoff, A. *Mol. Biol. Cell* **6**, 357–370 (1995).
- Shulga, N. *et al.* *J. Cell Biol.* (in the press).
- Altschul, S. F., Gish, W., Miller, W., Myer, E. W. & Lipman, D. J. *J. Mol. Biol.* **215**, 403–410 (1990).
- Fridell, R. A. *et al.* *Proc. Natl Acad. Sci. USA* **93**, 2936–2940 (1996).
- Meyer, B. E. & Malim, M. H. *Genes Dev.* **8**, 1538–1547 (1994).
- Yano, R., Oakes, M., Yamagishi, M., Dodd, J. A. & Nomura, M. *Mol. Cell. Biol.* **12**, 5640–5651 (1992).
- Moroi, Y., Hijioka, M., Blobel, G. & Radu, A. *Proc. Natl Acad. Sci. USA* **92**, 6532–6536 (1995).
- Chi, N. C., Adam, E. J. H. & Adam, S. A. *J. Cell Biol.* **130**, 265–274 (1995).
- Gorlich, D., Vogel, F., Mills, A. D., Hartmann, E. & Laskey, R. A. *Nature* **377**, 246–248 (1995).
- Malim, M. H., Bohnlein, S., Hauber, J. & Cullen, B. R. *Cell* **58**, 205–214 (1989).
- Zapp, M. L. & Green, M. R. *Nature* **342**, 714–716 (1989).
- Daly, T. J., Cook, K. S., Gray, G. S., Maione, T. E. & Rusche, J. R. *Nature* **342**, 816–819 (1989).
- Bohnelein, E., Berger, J. & Hauber, J. *J. Virol.* **65**, 7051–7055 (1991).
- Malim, M. H. & Cullen, B. R. *Cell* **65**, 241–248 (1991).
- Zapp, M. L., Hope, T. J., Parslow, T. G. & Green, M. R. *Proc. Natl Acad. Sci. USA* **88**, 7734–7738 (1991).
- Henriksen, R., Blobel, G. & Aris, J. P. *J. Biol. Chem.* **265**, 2209–2215 (1990).
- Iovine, M. K., Watkins, J. L. & Wente, S. R. *J. Cell Biol.* **131**, 1699–1713 (1995).
- Baudin, A., Ozier-Kalogeropoulos, O., Denouel, A., Lacroute, F. & Cullen, C. *Nucleic Acids Res.* **21**, 3329–3330 (1993).
- Hardy, C. F. *J. Mol. Cell. Biol.* **16**, 1832–1841 (1996).
- Yang, X., Hubbard, E. J. & Carlson, M. *Science* **257**, 68–72 (1992).

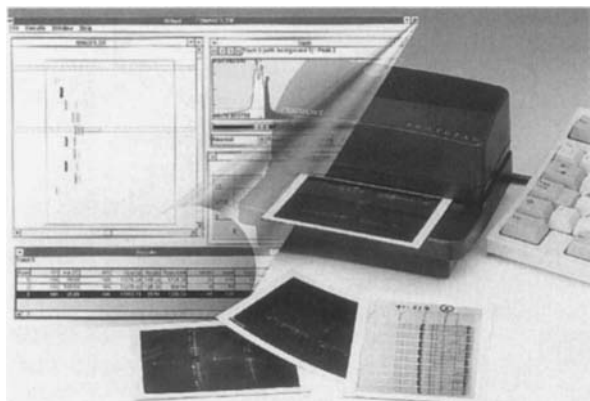
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**CORRESPONDENCE** and requests for materials should be addressed to S.R.W. (e-mail: swente@celbio.wustl.edu). The *GLE1* sequence has been deposited in the GenBank database, accession no. U68475.

# New laboratory technology

**This installment looks at equipment such as dispersion technology for particle size measurement, a homologous time-resolved assay system for drug discovery, a carbon fibre potentiostat and a robotic perfusion system.**

USING a Photopad scanner and UVigel analysis software from UVitec, users can efficiently and economically **analyse and document electrophoresis gels**. This system allows operators to plug the Photopad into the parallel port of a computer, install the software onto the hard drive and begin without complicated



**UVitec's Photopad gel scanner and UVigel software analyses electrophoresis gels.**

set-up procedures. After the print is fed into the Photopad it appears on the computer screen, where it can be analysed by the system's UVigel software. The software also allows the user to crop, rotate and enhance the image so that it can be printed on a laser or ink jet printer.

**Reader Enquiry No. 100**

The GlycoMap **electrophoresis system** from Oxford GlycoSystems has been developed specifically for high-throughput mapping of carbohydrates. It combines a custom-made electrophoresis unit, pre-cast carbohydrate gels, buffers, a fluorescent labelling kit and labelled standards. Three individual buffer systems, Tris-TAPS, Tris-Glycine and Tris-Tricine have been optimized for high-resolution electrophoresis of *N*-glycans, neutral *N*-glycans and *N*- and *O*-glycan mixtures, respectively. GlycoMap includes the Signal 2-AA fluorescent labelling kit for easy glycan labelling, allowing detection down to picomole levels. The fluorescent label, 2-aminobenzoic acid (2-AA), has a low molecular weight, a single negative charge and an intense fluorescent signal, making it particularly suitable as a label for carbohydrate electrophoresis. A single type of pre-cast gel is used for all buffer

systems, which is said to allow rapid, reproducible carbohydrate mapping. The electrophoresis unit has been designed with cooling from both sides of the gel providing high-resolution carbohydrate separations in only 90 minutes.

**Reader Enquiry No. 101**

Malvern Instruments' new dispersion technology for **particle size measurement** combats many of the problems associated with assessing the stability of disperse systems. The new product range includes the Zetasizer 3000, which is capable of measuring both zeta potential and particle size using the same sample in the same measurement cell. System features include a high-performance correlator and powerful laser, enabling rapid measurement of particles within the range of 2,000–3,000

nm. The company states that speed, accuracy and reproducibility are achieved through precise temperature control and pH measurement within the sample flow stream. A range of flow cells covers sample volumes from 100  $\mu$ l up to 2 ml, with a number of optional cells available for specific applications. Contamination between samples of highly absorbent, difficult to clean materials, such as surfactants, polymers and proteins, is eliminated by using a new low-volume disposable cell that requires only 0.7 ml of sample. Capillary cells and non-aqueous cells are specially designed for applications in high-resolution or high-salt conditions (for example samples in physiological saline) and for non-aqueous dispersants such as those found in toners and pigments. As an optional accessory, an Autosampler automates every stage of zeta potential and particle size measurement, achieving high throughput with excellent efficiency. An optional autotitrator is also available when a single zeta potential measurement is not enough, providing rapid and routine zeta potential profiles against changes in other parameters, such as pH or conductivity.

**Reader Enquiry No. 102**

A collaborative effort between Packard Instruments and Zeneca Pharmaceuticals has been established to develop a new **high-throughput assay system for drug discovery**. The result is homogeneous time-resolved fluorescence (HTRF), a new screening method that is designed to meet the increasing demands of screening laboratories for technologies that can facilitate high assay throughput. Based on the chemistry of Jean Marie Lehn, HTRF was developed by CIS Bio for the characterization of biomolecular interactions. Packard Instruments is offering HTRF to the life sciences along with Discovery, a high-capacity microplate analyser. The manufacturer says that the simple assay procedures associated with HTRF, together with the fast analysis time of Discovery, will allow automated throughputs of up to 50,000 samples per day. The system is being evaluated as part of an active program to increase the efficiency of HTRF by incorporating new assay technologies.

**Reader Enquiry No. 103**

## Microscopy



**Perkin-Elmer's FT-IR microscopy system with Image software.**

Perkin-Elmer's AutoImage system brings a new level of convenience to **Fourier transform-infrared (FT-IR) microscopy** with complete automation, interactive multimedia and enhanced performance characteristics. The system integrates a new microscope design with the capabilities of the company's Image software, which incorporates all microscope opera-



tions, including adjustments to aperture, focus and illumination, into a panel controlled on the computer screen. The system utilizes a Windows-based interface and is designed to provide consistent, reproducible data acquisition and access to new modes of operation, such as ATR for surface mapping studies. Key features of the system include autofocus, which allows, refocusing during unattended data collection operations, such as line scanning or mapping, and autoaperture, which enables users to select interactively the sampling area on the visible image. The software has different data collection modes to suit numerous application requirements. Data can be collected from a series of points, along a line or across a defined area.

## Reader Enquiry No. 104

Applied Imaging has added **digital, confocal capability** to the QuantCell 700 fluorescence imaging system. This combined hardware/software solution allows for the automated capture and deconvolution of images from multiple planes of focus, providing quality confocal images from a standard fluorescence microscope, appar-

ently dispensing with the need for expensive laser scanning modules. The process of acquiring multiple slices is now computer controlled and functions as a normal part of a fluorescence experiment. Setting the specification for a z-stack is no more complex than setting excitation or emission filters. Time-resolved three-dimensional fluorescence microscopy can now be achieved routinely, and confocal sets can be acquired in real time with deconvoluted data being interrogated in the same way as standard QuantiCell images. Interrogation options include three-dimensional intensity plotting, construction of histograms, plotting of line profiles and the extraction of time-resolved intensity data of any shape from multiple regions of interest. A powerful set of presentation options are also included. Confocal imaging is combined with the new 12-bit,  $1K \times 1K$  pixel camera with greater dynamic range, resulting in a system that is well suited for the accurate localization and quantification of multiple probes and widely varying degrees of brightness.

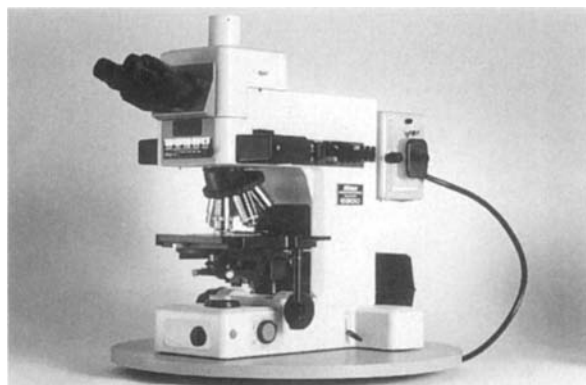
## Reader Enquiry No. 105

UltraViolet Products, manufacturer of the Pen-Ray Lamps, has announced the re-introduction of its **microscope replacement lamps**. These lamps are compatible with Nikon, Bausch & Lomb and Leica microscopes. The blue-white inspection lamps are phosphored at 445 nm for optimum visual results. The overall lamp diameter is 9.3 cm and the tube diameter is 1 cm.

## Reader Enquiry No. 106

Nikon has unveiled a new **research microscope system**, the Eclipse E800, which uses the CF160 infinity optical system to allow users to get sharp, clear images over a wide magnification range ( $\times 5 - \times 3,000$ ). This allows researchers to capture large images areas in film, digital and video formats. The CFI60's optical system offers the largest working distances the company has ever offered. The company has based this optical system on a new, longer objective parfocal length of 60 mm and a larger objective lens barrel with a diameter of 25 mm. The new system should be well suited for low light level applications such as fluorescence as it incorporates newly developed optical glass, cements and coatings for optimal light transmission. Researchers doing differential

interference contrast (DIC) work, video enhanced DIC, fluorescence *in situ* hybridization and other specialized techniques will find the large working distances useful for working with thick specimen sections. An additional asset to those doing multi-probe fluorescence work such as FISH is the new, five-position epi-fluorescence linear filter-cube slider, which makes changing



The E800 research microscope system from Nikon.

fluorescence cubes simpler. The new system also allows the user to document fields four times larger than with previous Nikon systems. The E800 system can document specimens at a 1:1 scale; a full 24-mm  $\times$  36-mm area of specimen can be captured on standard 35-mm film. Documentation is enhanced by using an easy-attaching photo system head, which allows the user to use four cameras at a time in various combinations, allowing the capture of 35-mm, CCTV, large-format photography and digital still photography images.

## Reader Enquiry No. 107

### Neuroscience tools

The Suprafusion 2500 **robotic perfusion systems** from Brandel are designed to stand up to the needs of *in vitro* neurotransmitter release studies. The new systems incorporate a variety of new features and include several new models, which offer automated programmable control, will perfuse tissues with up to nine reagents each and will collect up to 20 effluents per sample. The enhanced 20-channel model is now joined by models with 12 or 6 channels and will collect effluents into standard 5- or 7-ml vials in a generic collection tray. The new 18-channel model is designed for use with specific liquid scintillation counters where effluents are collected into vials that are preloaded into the counter's rack. Models are now available for use with Beckman, LKB and Packard counters, as well as many other types of counter. In addition, all models can now be supplied with an accessory that makes them suitable for perfusing larger tissue samples and performing other tests within well

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plates. These systems work with their own internal microprocessor and software, but can be interfaced to a host computer if necessary. Features offered as standard include heated reagent water baths and high-performance Versaflo peristaltic pumps.

**Reader Enquiry No. 108**

The MicroC, a new **carbon fibre potentiostat**, is now available from World Precision Instruments for the measurement of oxidizable biological compounds, such as adrenaline, noradrenaline, dopamine, serotonin, ascorbic acid. This ultra-sensitive, low-noise instrument detects redox currents ranging from 1 pA to 2  $\mu$ A while holding the working electrode at a constant voltage. The instrument has a rise time of less than 1.0 ms, and should prove useful in the study of quantized release of neurotransmitters. MicroC also features built-in carbon fibre-electrode activation, a low-pass filter and an optional external poise-voltage application. The potentiostat also has a connection to a chart recorder, oscilloscope or computer-based data-acquisition system.

**Reader Enquiry No. 109**

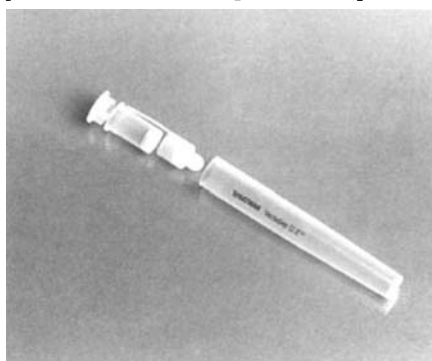
## Separation and analysis

Spectronics now offers a large selection of **ultraviolet transilluminators for gel electrophoresis**, chromatography, forensic science and more. The transilluminators provide improved performance by producing a brighter fluorescent response, higher clarity, as well as sensitivity down to one or two nanograms. These units are designed to ensure maximum protection against sample damage. All 312-nm and 365-nm transilluminators come equipped with a diffusing screen, which has been engineered to provide irradiance uniformity and to eliminate confusing light striations. The company's line of transilluminator products now includes 22 models to accommodate a variety of research requirements.

**Reader Enquiry No. 110**

Whatman has developed a new device for **centrifugal liquid extraction (CLE)** as a routine sample preparation technique. Designed to be simple to use, the new VectaSep extraction device is the company's first device to utilize centrifugal liquid extraction (CLE) for aqueous sample preparation in the analytical laboratory. It is manufactured in a ready-to-use format, and requires just two dispensing steps (solvent and sample) before extraction. The remaining steps are performed in only a few minutes. The extraction device is made from high-purity precision moulded polypropylene, is disposable and designed for use in 15-ml rotor cups within standard laboratory centrifuges. It comprises four parts: a

tube with separate cap, a sample disperser with integral microporous membrane and a separating cup. The manufacturer states that the extraction is a simple two-step process, involving the controlled extrusion of an aqueous sample from the through the dispersion



**Whatman's centrifugal extraction device.**

membrane into an extraction solvent by means of centrifugation. In the second stage, the sample disperser is replaced by a separating cup. A second, short centrifuge spin locates this cup at the solvent-sample junction, allowing analyses of interest to be selectively isolated.

**Reader Enquiry No. 111**

For concentrating DNA/RNA, proteins, amino acids in the field of molecular biology, Jouan offers **three centrifugal evaporators**: the RC 10.09, the RC 10.10 and the RC 10.22. Using vacuum, heat, air-pulse injection and centrifugation, these units rapidly concentrate the samples regardless of the type of solvent. The most commonly used 5-ml and 15-ml Eppendorf tubes fit into the easy handling rotors, which offer a capacity as high as  $202 \times 5$  ml tubes,  $4 \times 250$  ml tubes and two microtitre plates. A glass lid (RC 10.10, RC 10.22), ball bearing-free construction, central vapour outlet and three-way solenoid valve are some of the standard features offering protection from the corrosion of aggressive chemicals. (PTFE-coated chambers are also available.) There is, adjustable chamber heating, which has been optimized to avoid the risk of denaturation of samples, as well as save time. The RC 10.22 model offers a wide choice of programs, which can be stored and retrieved from memory. The air-pulse system reduces the evaporation time of aqueous solvents by up to 50 per cent.

**Reader Enquiry No. 112**

A new application note from Micromass is now available, covering **peptide analysis by capillary electrophoresis-mass spectrometry**: CE-ES-MS and CE-ES-MS-MS analysis of a standard peptide mixture. The technique has gained in popularity because of its low sample and solvent consumption, CE also is capable of high resolving power. Peptides, environmental

pollutants, drugs and surfactants are among the range of compound classes amenable to CE separations. Ultraviolet (UV) absorption is the standard detection method for CE, but the utility of the technique is enhanced greatly by mass spectrometric (MS) detection. Not only can molecular masses be accurately measured, but also the selectivity of tandem mass spectroscopy (MS-MS) allows generation of specific fragments from individual components in a mixture to verify or help deduce chemical structures. Electrospray (ES) ionization mass spectrometry is now considered the coupling method of choice for CE-MS, with the coaxial sheath flow probe as the most popular type of interface. With specific reference to a peptide mixture, this new literature demonstrates the simplicity of CE-MS and CE-MS-MS coupling, as well as the wealth of structural and molecular mass information readily achievable.

**Reader Enquiry No. 113**

## Labwares

Denley offers the use of HC refrigerants for its  $-85^\circ\text{C}$  range of **laboratory freezers**.

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**READER ENQUIRY NO. 651**

## PRODUCT REVIEW

ers. According to the manufacturer, replacing CFC with HC results in lower energy consumption and reduces the risk of atmospheric pollution. These freezers are design with a small footprint, and still maintaining capacities from 226 l to 549 l cubic feet. To safeguard precious samples, ultra-low freezers are equipped with the latest in audio-visual alarms and offer full chart recording, security autodialer and temperature logging options.

**Reader Enquiry No. 114**

Labconco now offers the Paramount **filtered enclosure**, which is designed to remove small quantities of contaminants, such as organic solvents, acid gases, formaldehyde and ammonia. As an internal blower pulls air into the enclosure, contaminants released within are diluted



**The Paramount filtered enclosure from Labconco is portable and saves energy.**

by the air, drawn through a rear baffle and adsorbed onto, or treated by, two internal carbon filters. A front air foil directs air flow into the enclosure to minimize turbulence and to maximize fume containment. As the cabinet requires no ducting, it is movable, conserves energy and has lower installation costs than traditional fume hoods. It should also be useful in 'air-starved' laboratories.

**Reader Enquiry No. 115**

Robbins Scientific has released the Gemini **twin shaking waterbath**, composed of two independently controlled heated chambers for running two procedures simultaneously. The low-profile chamber design was engineered to provide easy access to incubation containers for adding or removing reagents. Removable stainless steel chamber adapters hold vessels such as Tupperware containers, microplates and trays firmly in place during incubation without the use of lead weights. Small and lightweight, the Gemini has a footprint of 33 cm x 50 cm and weighs 14 kg. Its heating system

reaches the desired temperature within 25 min and enables the unit to recover to the set temperature in under 20 s after the cover has been removed. A clear acrylic cover allows the operator to change reagents quickly and to see the samples during processing. Agitation to both chambers is provided as a front-to-back reciprocating motion and is adjustable from 10 to 160 oscillations per minute.

**Reader Enquiry No. 116**

Composite Rotors has developed OMP-spin **carbon-fibre rotors** for high-speed and ultra-centrifuges from will generate force fields in excess of 48,000g. In ultra-centrifuges these rotors will achieve maximum speeds from 78,000g to 100,000g. Removable anodized aluminium tube holders are available to spin 50-ml round bottomed, screw-cap bottles in both rotor models. Open-top tube holders are used for standard operation, while sealed-type tube holders are available for processing pathogenic samples. At maximum rotor speeds sample volumes of 13.5 ml, 15 ml and 38 ml can be processed in designated tube adapters. The rotors will also spin 24 or 36 x 1.5-ml microcentrifuge tubes for isolation of small amounts of plasmid DNA, cytosol fractions and membrane fragments at maximum rotor speeds. These rotors use conical tube holders for 50 ml culture tubes instead of requiring the use of a special rotor. Holders can be used in these rotors to collect bacterial or mammalian cells from cultures at 12,000 r.p.m. (20,000g).

**Reader Enquiry No. 117**

Beckman has recently redesigned its Optima series of **floor standing and table-top ultra-centrifuges**, with the intent to improve performance and ergonomics. The new models comprise the Optima XL-100 K, the L-90 K, the LE-80 K and the bench-top Optima TL-TLX. Two new rotors, the NVT 100 and the Type 100 Ti, have been introduced for use with floor standing Optima K series models. This series of ultra-centrifuges have been designed to offer reliability, versatility and safety, as well as meet world-wide standards for quality and operator safety, certified by the CE mark, UL and CSA approvals. The centrifuges feature intuitive microprocessor-controlled operation, as well as an air-cooled induction drive system and a thermoelectric temperature control that is designed to eliminate the need for a compressor, thereby reducing power requirements.

**Reader Enquiry No. 118**

Over two dozen different Hanna **meters and laboratory measuring instruments** are available from Sigma Chemical Company. Included is a broad selection of pH meters, conductivity meters and thermometers. The company also offers an

oxygen meter, a thermohygrometer and an electrode simulator. A wireless infrared computer interface for Hanna dataloggers is also available. Fifteen different pH meters are available, including models with automatic calibration, built-in thermometers, built-in printers, and datalogging capability. Among the six conductivity meters are units for general laboratory use as well as datalogging models.

**Reader Enquiry No. 119**

*These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.*

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**READER ENQUIRY NO. 81**